AD_____

Award Number DAMD17-96-1-6046

TITLE: 1996 International Conference on Biomedical Aspects of Clostridial Neurotoxins

PRINCIPAL INVESTIGATOR: Keith A. Foster, Ph.D.

CONTRACTING ORGANIZATION: Centre for Applied Microbiology and Research
Wiltshire SP4 OJG, United Kingdom

REPORT DATE: June 1997

TYPE OF REPORT: Final Proceedings

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 1997	Final Proceedings (15 Apr 96 - 14 Apr 97)

4. TITLE AND SUBTITLE
1996 International Conference on Biomedical Aspects of Clostridial Neurotoxins

5. FUNDING NUMBERS
DAMD17-96-1-6046

6. AUTHOR(S)
Keith A. Foster, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Centre for Applied Microbiology and Research
Wiltshire SP4 OJG, United Kingdom

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

14. SUBJECT TERMS

15. NUMBER OF PAGES
166

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Oxford (UK) Conference 1996
8th July–11th July

Biomedical Aspects of Clostridial Neurotoxins

Edited by

Howard S Tranter PhD
Centre for Applied Microbiology and Research
Porton Down, Salisbury, Wiltshire SP4 0JG, UK

CAMR

Centre this photo

Delegates at the Oxford (UK) Conference 1996 on Biomedical Aspects and Clostridial Neurotoxins

Contents

Session 1

Organisms and Disease

Epidemiology of foodborne botulism

Roberts, T.A.

Food Safety Consultant, 59 Edenham Crescent, Reading RG1 6HU.

Introduction

Clostridium botulinum was defined by Prévot (1953) as the species designation for all organisms producing botulinum neurotoxin, currently comprising seven serologically distinct toxin types (A-G). A toxin type may not be restricted to one phenotypic group of organisms (see Collins, this Symposium). The most important groups in foodborne botulism in man are Group I (proteolytic, mesophilic) and Group II (non-proteolytic/saccharolytic, psychrotrophic). The taxonomic status of the *C. botulinum* group, based on phenotype, was reviewed by Hatheway (1993). The relationships of the various types of *C. botulinum* and other clostridia capable of producing botulinum neurotoxin to other clostridia, based on 16S rRNA sequencing, have been explored by Lawson *et al.* (1993).

Four different forms of botulism are recognized. If *C. botulinum* multiplies in a food, and that food is subsequently ingested without heating, the consumer of that food suffers botulism *via* **intoxication** from preformed toxin. There are reports that another form of botulism occurs when *C. botulinum* multiplies in the gut, producing neurotoxin and causing a "**toxico-infection**" (see Soviet papers in Ingram and Roberts, 1967; Chia *et al.*, 1979; McCroskey and Hatheway, 1988). In **wound botulism**, *C. botulinum* establishes itself and multiplies in damaged tissues, and in doing so produces neurotoxin which is absorbed, causing botulism (Merson and Dowell, 1973). The fourth form is **infant botulism** when multiplication occurs in the gut of the young (Chin *et al.*, 1986; Arnon, this Symposium).

Confirmation of botulism should include demonstration of toxicity e.g. in mice, displaying the typical symptoms of partial posterior paralysis, characteristic laboured diaphragmatic breathing and "wasp waist". That toxicity should be neutralized by polyvalent, and ideally monovalent, antitoxins. In addition, the toxicity should be destroyed by heating e.g. boiling for 2 min (botulinum toxin is destroyed but tetanus toxin is not). In practice, the levels of neurotoxin in the serum of the affected person (or animal) are often very low (2 MLD/ml), so it has become common also to test faeces for toxin and for the presence of *C. botulinum*.

The mouse test has proved unreliable with heavily spoiled foods, and on enrichment cultures from material that is initially heavily contaminated with other microorganisms, because toxic compounds other than botulinum toxin may be present. Various procedures have been suggested to minimise their effects which confuse the symptoms of botulism in the test mice, including dilution, freezing, immunizing the mice with immune sera against other clostridia, and injecting mice with antibiotics such as streptomycin, tetracycline. Occasionally, even if samples are centrifuged or filtered to remove bacterial cells, mice suffer infections that make it difficult or impossible to confirm the diagnosis of botulism.

C. botulinum has the capacity to affect essentially all vertebrates (Table 1). Man is most commonly affected by types A, B and E, with a few reports of type F. Types C and D have been suspected of causing human botulism and reported in the literature, but by the criteria applied today, types C and D botulism have not been confirmed in man (discussed in Roberts and Gibson, 1979; Hatheway, 1993). While surveying soil in Argentina, Gimenez and Ciccarelli (1970) isolated an organism that produced a toxin able to kill mice with symptoms indistinguishable from those of botulism, but not neutralized by any monovalent or polyvalent *C. botulinum* antitoxins. This was named *C.botulinum* type G but has since been reclassified as *C. argentinense* (Suen *et al.*, 1988). Although type G was isolated retrospectively after sudden death in adults (Sonnabend *et al.*, 1981) and infants (Sonnabend *et al.*, 1985) doubt remains that it was the cause of the deaths (Hatheway, 1993). Type G has never been demonstrated in, or isolated from, foods.

C. botulinum can be isolated from soil, mud and aquatic sediments, the ease or difficulty of isolation varying with the geographical region. It is relatively common in some raw foods e.g. Baltic herring

Table 1. Botulism toxins produced by *Clostridium botulinum*

Type	Species affected	Vehicles
A	Man (also wound and infant) Chickens ("limberneck")	Home-canned vegetables, fruits, meat, fish
B	Man (also wound and infant) cattle, horses	Prepared meats (esp. pork)
Ca	Aquatic wild birds ("Western duck sickness")	Toxin-bearing invertebrates, Carrion, forage
Cb	Cattle ("Midland cattle disease" Horses ("forage poisoning") Mink	
D	Cattle ("lamziekte")	Carrion
E	Man	Uncooked products of fish and marine mammals
F	Man	Home-made liver paste "deer-jerky"
G (*C. argentinense*)	Unknown (man ?)	?

(Johannsen, 1963) , Pacific salmon, trout farmed in ponds with earth bottoms (Bach and Muller Prasuhn, 1971; Huss *et al.*, 1974a,b), pork (Roberts and Smart, 1976, 1977; Tompkin, 1980), mushrooms (Hauschild *et al.*, 1975). It has also been isolated from fruit, vegetables, onion skins and garlic cloves (Solomon and Kautter, 1986, 1988), cabbage, spinach (Insalata *et al.*, 1970), honey (Sugiyama *et al.*, 1978; Midura *et al.*, 1979) but not corn syrup (Lilly *et al.*, 1991).

No means are known to prevent, or control, the frequency of contamination, so food processors assume spores of *C. botulinum* may be present and adjust processing to destroy spores, or preservation to prevent toxin production (see McClure, this Symposium).

The most complete early records of human botulism were from the USA, collated in California. Types A and B were most common (Table 2), but many cases of suspected botulism could not be confirmed. Prospects for patient recovery were initially poor but have improved with respiration maintained artificially.

When available figures were broken down into those resulting from commercial foods or due to home processing of foods, the latter was the more common, usually traced to a poor understanding of the principles of food preservation e.g. inadequate thermal process in home canning / home bottling (Table 3). Underprocessing, particularly of low-acid produce by home canners, has been a major source of botulism from vegetables. For example, in the USA between 1899 and 1977, 180 of 297 outbreaks were attributed to vegetables (Anon., 1979; Roberts *et al.*, 1982). The major proportion were attributed to string beans, other produce included peppers, potatoes, beans, beets, mushrooms, corn, carrots, olives and celery.

The foods implicated in foodborne botulism (Table 4) illustrate the widespread contamination of raw agricultural products of all types. Detailed information on numerous outbreaks is conveniently summarized by Rhodehamel *et al.* (1992).

Hauschild and Dodds (1993) edited an excellent overview of all aspects of the ecology and control of *C. botulinum* in foods, including much detailed epidemiology not previously available. Tables 5-9 below are summarized from Hauschild (1993).

Available statistics reveal that some countries report foodborne botulism more frequently than others (compare Tables 5, 6 and 7)

Hauschild (1993) also provided information on the foods most commonly associated with botulism in each country (Table 8), including whether the food

Table 2. Botulism in the USA 1899-1977

C. botulinum	cases	deaths	%
type A	576	304	53
type B	228	66	29
type E	91	30	33
type F	3	0	0
mixed A and B	6	2	33
type unknown	1057	597	56
total	1961	999	51

Table 3. Outbreaks of botulism in the USA (1899-1977)

Total outbreak	766
Home prepared/home canned	548
Commercially processed	66
Unknown	152

Table 4. Foods associated with foodborne botulism in the USA (1899–1977)

	Type						
	A	B	E	F	A and B	Unknown	Total
Vegetables	115	31	1	-	2	2	151
Fruits	22	7	-	-	-	-	29
Beef, venison, mutton	6	1	-	1	-	-	8
Pork	2	1	-	-	-	-	3
Poultry	2	2	-	-	-	-	4
Fish and products	11	4	25	-	-	1	41
Milk and products	3	2	-	-	-	-	5
Condiments	17	5	-	-	-	1	23
Others	8	3	3	-	-	-	14
Unknown	9	3	1	-	-	6	19
Total							297

Table 5. Countries with recorded cases of foodborne botulism

Alaska	Argentina	Belgium	Canada
China	Czechoslovakia	Denmark	France
Germany	Hungary	Iran	Italy
Japan	Norway	Poland	Portugal
Spain	USA	USSR	Yugoslavia

(adapted from Hauschild, 1993)

Table 6. Countries with fewer than 7 recorded outbreaks

Australia (5)	Brazil (4)	Chile (1)
Finland (1)	Guatemala (6)	Iceland (2)
Israel (1)	Kenya (2)	Madagascar (2)
Mexico (2)	Netherlands (2)	New Zealand (1)
Peru (1)	Sweden (6)	Switzerland (5)
Taiwan (5)	UK (3)	

(*) number of reported cases (adapted from Hauschild, 1993)

Table 7. Countries with no reports of human foodborne botulism

Austria	Greece	India
Ireland	Nigeria	Venezuela

(from Hauschild, 1993)

was home- or commercially-produced, and the serotypes of *C. botulinum* most commonly causing illness in each country (Table 9).

Foodborne botulism most commonly occurs because of an inadequate process and/or inappropriate storage conditions. For example, botulism in Japan, the Baltic counties, among Eskimos in Canada and Alaska still occurs from traditional fermented fish or fermented marine products (Dolman, 1964). The raw ingredients naturally carry psychrotrophic strains of *C. botulinum* and preventing their multiplication is dependent on a rapid fall in pH value in the early stages of fermentation and maturation. The fermentation occurs "naturally" and is not controlled. Similarly, in Europe hams produced on farms have caused botulism because the curing process (the application of salt and sodium nitrite) was not under control.

Botulism in circumstances that might have been expected

After an outbreak of botulism the reasons for the outbreak often become obvious. For example, fatal botulism from commercially canned salmon was traced to defects in cans that, coupled with poor hygienic practices in the cannery, resulted in post-processing contamination of the food by type E spores. Botulism from hazelnut yoghurt (O'Mahoney *et al.*, 1990) was traced to the canned nut purée not having received an adequate heat treatment, and possibly replacement of sugar in the product by a sweetener.

There are numerous accounts of botulism arising

Table 8. Foods involved in outbreaks of botulism

	Outbreaks with food identified	food (%)				food source home (%)
		meats	fish	fruit and vegetables	other	
USA	222	16	17	59	9	92
Canada	75	72	20	8	0	96
Argentina	14	29	21	36	14	79
Poland	83	12	5	0	75	
Czechoslovakia	14	72	7	14	7	100
Germany						
East	31	52	26	19	3	73
West	55	78	13	9	0	100
Belgium	8	75	12	12	0	62

(adapted from Hauschild, 1993)

Table 9. *C. botulinum* types involved in outbreaks

	Outbreaks with type identified	type (%)			
		A	B	E	other
USA	252	61	21	17	0.4 (F)
Canada	76	4	8	88	0
Argentina	13	77	8	0	15 (A – F, AF)
Poland		3	94	3	0
Czechoslovakia	6	17	83	0	0
Hungary	31	0	100	0	0
Germany			> 90 (estimated)		
Belgium	11	0	55	9	36 (B – C)
France	171	0	97	2	0.6 (AB)
Italy	15	20	60	7	13 (A – B)
Spain	36	0	92	3	6 (A – B)
Portugal	18	0	100	0	0
Denmark	11	0	0	100	0
Norway	19	0	47	47	5 (F)
USSR	45	33	38	29	0
Iran	63	0	3	97	0
China	733	93	5	1	0.4 (A – B)
Japan	97	2	2	96	0

(from Hauschild, 1993)

from inadequately home-processed processed green beans, asparagus, beets, jalapeno peppers (Terranova *et al.*, 1978).

A large outbreak of botulism, including fatalities, occurred in Cairo, Egypt from "faseikh": fermented, fresh, uneviscerated mullet, subsequently dry-salted in barrels and normally consumed without cooking. Kapchunka (commercially produced, ready to eat, salt cured, air dried uneviscerated whitefish) has also been responsible for botulism.

Botulism in circumstances that were surprising.

There have also been several outbreaks of botulism in circumstances that were unusual, even unexpected.

Although huge quantities of cheese are prepared worldwide using traditional processes and technologies, its record of microbiological safety has been good. However, Brie cheese was believed to be responsible for type B botulism in Switzerland and

France (Sébald *et al.*, 1974), and a cheese spread containing onion was implicated in botulism in Argentina (de Lágarde, 1974). Further details are given in Collins-Thompson and Wood (1993).

Potato salad prepared from cooked foil-wrapped potatoes that were allowed to cool slowly and were not refrigerated over-night caused botulism (Seals *et al.*, 1981; Brent *et al.*, 1995) as have sautéed onions (MacDonald *et al.*, 1985).

C. botulinum is able to multiply on the surface of mushrooms overwrapped in retail packs, unless care is taken to allow accumulated respiratory gases to escape (Kautter *et al.*, 1978). Chopped garlic in soybean oil, with pH >4.6 and stored unrefrigerated, was responsible for a large outbreak of (type B) botulism in Canada (Health and Welfare Canada, 1986; FDA, 1989).

Commercially preserved peanuts were implicated in botulism in China (Chou *et al.*, 1988) and vacuum-packed lotus rhizome in another outbreak (Otofuji *et al.*, 1987).

Botulism has also been associated with black olives (Fenicia *et al.*, 1992) and roasted eggplant in oil (D'Argenio *et al.*, 1995).

Such examples indicate that contamination of the agricultural environment with *C. botulinum* is inevitable, and there are many opportunities for it to multiply and produce sufficient toxin to cause botulism if those products are not handled and stored under appropriate conditions. Although an anaerobe by definition, sufficiently anaerobic conditions exist in many foods for the possible growth of *C. botulinum* to be taken seriously.

References

Anon (1979) Botulism in the United States, 1899-1977. A Handbook for Epidemiologists, Clinicians and Laboratory Workers, May 1979. Center for Disease Control, Atlanta, GA, USA.

Bach R and Muller Prasuhn G (1971) Farmed trout as carrier of *Clostridium botulinum* and cause of botulism. I. Literature survey on *Clostridium botulinum* type E and fish botulism. *Arch Lebensmittelhyg* **22**, 64-68.

Brent J, Gomez H, Judson F, Miller K, Rossi-Davis A, Shillam P, Hatheway C, McCrosky L, Mintz E, Kallander K, McKee C, Romer J, Singleton E, Yager J and Sofos J (1995) Botulism from potato salad. *J. Food Prot.* **15**, 420-422.

Chia JK, Clark JB Ryan CA and Pollack M (1986) Botulism in an adult associated with food-borne intestinal infection with *Clostridium botulinum*. *New Engl. J. Med.* **315**, 239-241.

Chin J, Arnon SS and Midura TF (1979) Food and environmental aspects of infant botulism in California. *Rev. Infect. Dis.* **1**, 693-696.

Chou JH, Hwang PH and Malison MD (1988) An outbreak of type A foodborne botulism in Taiwan due to commercially preserved peanuts. *Int. J.Epidemiol.* **17**, 899-902.

Collins-Thompson DL and Wood DS (1993) Control in dairy products. In Hauschild AHW and Dodds KL (eds) *Clostridium botulinum: Ecology and control in foods*. Marcel Dekker, New York; pp 261-277.

D'Argenio P, Palumbo F, Ortolani R, Pizzuti R, Russo M, Carducci R, Soscia M, Aureli P, Fenicia L, Franciosa G, Parella A and Scala V (1995) Type B botulism associated with roasted eggplant in oil – Italy, 1993. *Morbid. Mortal. Weekly Rep.* **44**, 33-36.

de Lágarde EA (1974) Boletin informativo del Centre Panamericano de Zoonosis, Vol. 1 Centro Panamericano de Zoonosis, Buenos Aires, Argentina.

Dolman CE (1964) Botulism as a work health problem. In Botulism : Proceedings of Symposium, Cincinnati, Ohio. Public Health Service Publication No. 999-FP-1, US Department of Health, Education and Welfare, Cincinnati, Ohio; pp 43-68.

Fenicia L, Ferrini AM, Aureli P and Padovan MT (1992) Botulism outbreak from black olives (in Italian). *Industrie Aliment.* **31**, 307-308.

FDA (1989) HHS News, P89-20, United States Food and Drug Administration, Washington DC, USA.

Gimenez DF and Ciccarelli AS (1970) Another type of *Clostridium botulinum*. *Zentralbl. Bakteriol. Ab.t Orig. A* **215**, 221-224.

Hatheway CL (1993) *Clostridium botulinum* and other clostridia that produce botulinum neurotoxin. In Hauschild AHW and Dodds KL (eds) : *Clostridium botulinum: Ecology and Control in Food*. Marcel Dekker Inc, New York; pp3-20.

Hauschild AHW, Aris BJ and Hilsheimer R (1975) *Clostridium botulinum* in marinated products. *Can. Inst. Food Sci. Technol. J.* **8**, 84.

Hauschild AHW (1993) Epidemiology of human foodborne botulism. In Hauschild AHW and Dodds KL (eds) : *Clostridium botulinum: Ecology and Control in Food*. Marcel Dekker Inc, New York; pp69-104.

Hauschild AHW and Dodds KL (eds) (1993) *Clostridium botulinum: Ecology and Control in Foods*. Marcel Dekker, New York.

Health and Welfare Canada (1986) Botulism in Canada – summary for 1985. *Canadian Diseases Weekly Reports* **12**, 53-54.

Health and Welfare Canada (1987) Restaurant-associated botulism from in-house bottled mushrooms – British Columbia. *Canadian Diseases Weekly Reports* **13**, 35-36.

Huss HH, Pederson A and Cann DC (1974a) The incidence of *Clostridium botulinum* in Danish trout farms. I. distribution in fish and their environmental. *J. Food Technol.* **9**, 445-450.

Huss HH, Pederson A and Cann DC (1974b) The incidence of *Clostridium botulinum* in Danish trout farms. II. measures to reduce contamination of the fish. *J. Food Technol.* **9**, 451-458.

Ingram M and Roberts TA (1967) *Botulism 1966*. In Ingram M and Roberts TA (eds): Proceedings of the 5th International Symposium on Food Microbiology. Chapman and Hall, London.

Insalata NF, Witzeman SJ and Berman JH (1970) The problems and results of an incidence study of the spores of *Clostridium botulinum* in frozen vacuum-pouch-pack vegetables. *Devel. Ind. Microbiol.* **11**, 330-334.

Johannsen A (1963) *Clostridium botulinum* in Sweden and the adjacent waters. *J. Appl. Bact.* **26**, 43-47.

Kautter DA, Lilly T Jr and Lynt R (1978) Evaluation of the botulism hazard in fresh mushrooms wrapped in commercial polyvinylchloride film. *J. Food Prot.* **41**, 120-121.

Lawson PA, Llop-Perez P, Hutson RA, Hippe H and Collins M.D (1993) Towards a phylogeny of the clostridia based on 16S rRNA sequences. *FEMS Microbiol. Lett.* **113**, 87-92.

Lilly T Jr ,Rhodehamel EJ, Kautter DA and Solomon HM (1991) *Clostridium botulinum* spores in corn syrup and other syrups. *J. Food Protect.* **54**, 585-587.

MacDonald KL , Spengler RF, Hatheway CL, Hargrett NT and Cohen ML (1985) Type A botulism from sauteed onions. **JAMA 253**, 1275 -1278.

MacDonald KL, Cohen ML and Blake PA (1986) The changing epidemiology of adult botulism in the United States. *Am. J. Epidemiol.* **124**, 794-799.

McCroskey LM and Hatheway CL (1988) Laboratory findings in four cases of adult botulism suggest colonization of the intestinal tract. *J. Clin. Microbiol.* **26**, 1052-1054.

Merson MH and Dowell VR Jr (1973) Epidemiological, clinical and laboratory aspects of wound botulism. *N. Engl. J. Med.* **289**, 1005-1010.

Midura TF, Snowden S, Wood RM and Arnon SS (1979) Isolation

of *Clostridium botulinum* from honey. *J. Clin. Microbiol.* **9**, 282-283.

O'Mahony M, Mitchell E, Gilbert RJ, Hutchinson DN, Begg NT, Rodhouse JC and Morris JE (1990) An outbreak of foodborne botulism associated with contaminated hazelnut yogurt. *Epidemiol. Infect.* **104**, 389-395.

Otofuji T, Tokiwa H and K Takahashi (1987) A food poisoning incident caused by *Clostridium botulinum* toxin A in Japan. *Epidemiol. Infect.* **99**, 167-172.

Prévot AR (1953) Rapport d'introduction du Président du Sous-Comité *Clostridium* pour l'unification de la nomenclature des types toxigeniques de *C. botulinum*. *Int. Bull. Bacteriol. Nomencl. Taxon.* **3**, 120-123.

Rhodehamel EJ, Reddy NR and Pierson MD (1992) Botulism: the causative agent and its control in foods. *Food Control* **3**, 125-143.

Roberts TA and Smart JL (1976) The occurrence and growth of *Clostridium* spp. in vacuum-packed bacon with particular reference to *Clostridium perfringens (welchii)* and *Clostridium botulinum*. *J. Food Technol.* **11**, 229-244.

Roberts TA and Smart JL (1977) The occurrence of clostridia, particularly *Clostridium botulinum*, in bacon and pork. In Wolf J Barker AN Ellar DJ Dring GJ and Gould GW (eds): *Spores 1976*. Academic Press, London; pp 911-915.

Roberts TA and Gibson AM (1979) The relevance of *Clostridium botulinum* type C in public health and food processing. *J. Food Technol.* **14**, 211-226.

Roberts D, Watson GN and Gilbert RJ (1982) Contamination of food plants and plant products with bacteria of public health concern. In Rhodes-Roberts ME and Skinner FA (eds) : *Bacteria and Plants*. Academic Press, London; pp169-195.

Seals JE, Snyder JD, Edell TA, Hatheway CL, Johnson CJ and Hughes JM (1981) Restaurant-associated type A botulism: transmission by potato salad. *Am. J. Epidemiol.* **113**, 436-444.

Sébald M, Jouglard J and Gilles G (1974) Botulisme humaine de type B aprés ingestion de fromage. *Ann. Microbiol.* **125A**, 349-357.

Solomon HM and Kautter DA (1986) Growth and toxin production by *Clostridium botulinum* in sauteed onions. *J. Food Prot.* **49**, 618-620.

Solomon HM and Kautter DA (1988) Outgrowth and toxin production by *Clostridium botulinum* in bottled chopped garlic. *J. Food Prot.* **51**, 862-865.

Solomon HM, Kautter DA, Lilly T and Rhodehamel EJ (1990) Outgrowth of *Clostridium botulinum* in shredded cabbage at room temperature under a modified atmosphere. *J. Food Prot.* **53**, 831-833, 845.

Sonnabend O, Sonnabend W, Heinzle R, Sigrist T, Dirnhofer R and Krech U (1981) Isolation of *Clostridium botulinum* type G and identification of type G botulinal toxin in huamns: report of five sudden unexpected deaths. *J. Infect. Dis.* **143**, 22-27.

Sonnabend OAR, Sonnabend WFF, Krech V, Molz G and Sigrist T (1985) Continuous microbiological and pathological study of 70 sudden and unexpected infant deaths. Toxigenic intestinal *Clostridium botulinum* infection in 9 cases of sudden infant death syndrome. *Lancet* **i**, 237-241.

St. Louis ME, Peck SHS, Bowering D, Morgan GB, Blatherwick J, Banerjee S, Kettyls GDM, Black WA, Milling ME, Hauschild AHW, Tauxe RV and Blake PA (1988) Botulism from chopped garlic: delayed recognition of a major outbreak. *Ann. Int. Med.* **108**, 363-368.

Suen JC, Hatheway CL, Steigerwalt AG and Brenner DJ (1988) *Clostridium argentinense* sp. nov.: a genetically homogeneous group composed of all strains of *Clostridium botulinum* type G and some non-toxigenic strains previously identified as *Clostridium subterminale* or *Clostridium hastiforme*. *Int. J. Sys. Bacteriol.* **38**, 374-381.

Sugiyama H, Mills DC and Kuo L-JC (1978) Number of *Clostridium botulinum* spores in honey. *J. Food Prot.* **41**, 848-850.

Terranova W, Breman JG, Locey RP and Speck S (1978) Botulism type B: Epidemiologic aspects of an extensive outbreak. *Am. J. Epidemiol.* **108**, 150.

Tompkin RB (1980) Botulism from meat and poultry products a historical perspective. *Food Technol.* **34**, 229-236, 259.

Control of *Clostridium botulinum* in food processing

Peter J. M^{c}Clure

Unilever Research, Colworth House, Sharnbrook, Beds., MK44 2DB, UK.

Key words: *Clostridium botulinum,* preservation, thermal inactivation, predictive modelling, control, spores

Clostridium botulinum has been the principle target microorganism in food processing for more than 70 years. In order to prevent foodborne botulism occurring: the food product/processing operation/container should be kept free from contaminating cells/spores; any contaminating spores/cells should be destroyed during processing or spore germination/outgrowth and toxin production in product should be prevented. In practice, the main methods of control fall into the latter two categories *i.e.* elimination of the organism or control of growth/toxin production. The differences between proteolytic and non-proteolytic strains of *C. botulinum* result in control of different process requirements. The most common method for the preservation of low acid, hermetically sealed foods, has been the application of heat. An overview of the main methods of control is presented. Good control requires accurate descriptions of rates of spore inactivation or growth inhibition over a wide range of conditions. Approaches are described which can take account of these factors without compromising safety.

Introduction

The causative agent of botulism was first isolated by Van Ermengem (1897) from an outbreak in Holland caused by consumption of raw salted pork, and first named *Bacillus botulinus*. Cases of foodborne disease showing classic symptoms of botulism were, however, recorded as early as 1820. The species *C. botulinum* is divided into seven types, of which types A, B, E and F are normally associated with human foodborne botulism. These types are classified into four distinct groups, based on cultural and serological characteristics: group I includes proteolytic types A, B and F; group II contains all type E strains and the non-proteolytic strains of types B and F; group III contains types C and D strains normally associated with animal botulism; group IV contains the proteolytic (non-saccharolytic) type G strains. To cause botulism, cells or spores must first contaminate a food and survive the food processing treatment or contaminate the food after processing, and then grow and produce toxin in the food. The food must be consumed without sufficient heating to destroy the toxin. The physiological differences between groups I and II result in these strains behaving differently in response to factors commonly used in foods to prevent their growth and toxin formation, or to inactivate them. Generally speaking, proteolytic strains are more resistant to physical and chemical agents, whilst the non-proteolytic strains can grow at lower temperatures. Hence, proteolytic strains are a major concern in the processing of low acid canned foods, whilst non-proteolytic strains cause problems primarily in pasteurized or unheated foods, including seafood.

Inactivation

Although there are a number of methods which may be used to inactivate spores, including exposure to chlorine compounds, ethylene oxide, hydrogen peroxide, and ionizing radiation, the most commonly used method is thermal inactivation. This was first introduced by Nicholas Appert in 1810, when he developed the first heat treated hermetically sealed containers, for the French Army, Navy and Merchant Marine. Typical heat treatments recommended for these foods were between 15 min and 2.5 h in boiling water and in comparison to existing processes, these heat treatments would not be sufficient to provide adequate safety. The process which is used today is commonly referred to as the 12-D botulinum cook. This process is based on the definitive study by Esty and Meyer (1922) where they examined the heat resistance of 108 strains of *C. botulinum*. The majority of this work was carried out in phosphate buffer because of poor reproducibility observed in foods.

This study is often quoted as the first study to demonstrate logarithmic survival, and forms the basis of the canning industry standard D value of 0.2 minutes at 121°C. There are, however, many examples of deviations from logarithmic order of death, for which various explanations have been suggested (Cerf, 1977). There are a number of factors which can influence the heat resistance of bacterial spores (see Kim and Foegeding, 1992): temperature and presence of divalent cations and fatty acids during sporulation; presence of fats/lipids, pH, acidulant, and water activity during heating; and recovery conditions. Non-proteolytic *C. botulinum* spores have been shown to have a greater apparent heat resistance if recovered in the presence of lysozyme (Peck and Fernandez, 1995). It is thought that enzymes required for germination are destroyed during heating and their function is provided by lysozyme added to the recovery medium. This may be important in foods which contain such enzymes, since they may facilitate germination of spores which are not destroyed by the heat process.

Despite there being a wide range of factors which may influence heat resistance of spores, the canning industry has an excellent safety record (Odlaug and Pflug, 1978). There have, however, been a number of outbreaks due to commercially prepared foods, usually as a result of process failure or post-process contamination (see M°Clure *et al.*, 1994). Table 1 summarises limits for growth and values of inactivation.

Prevention of growth and toxin production

C. botulinum can be controlled in foods without having to resort to severe heat treatments which often reduce nutritional and organoleptic acceptability. Since the organism must be able to germinate and multiply in a food to produce toxin and cause disease, inhibition of germination and growth can be achieved by controlling one or more factors such as pH, water activity, temperature or presence of preservatives. The efficacy of antimicrobial factors, as with heat treatment, will depend on other conditions in the food environment, and combinations of factors are often used where a single factor acting alone would not be sufficient.

pH

It is generally accepted that *C. botulinum* will not grow or produce toxin below pH 4.6. Nevertheless, there are a number of studies (Smelt *et al.*, 1982; Tanaka, 1982; Wong *et al.*, 1988) which have demonstrated growth below 4.6, invariably in the presence of high concentrations of protein and particular acidulants. It is thought that the protein provides microenvironments with local pH values higher than those that are measured, or that the protein provides a buffering effect or essential nutrient for growth. The regulations for acid canned fruit and vegetables require that the pH is less than 4.6, since there is relatively little protein likely to be present.

There is good evidence (Townsend *et al.*, 1954; Baird-Parker and Freame, 1967) that *C. botulinum* will not grow and produce toxin in acidified fruits and vegetables, but there are a number of documented cases of botulism resulting from these products, the majority of which are home canned. These result from failure of process design, process delivery or post-process contamination. There is also evidence that growth of other organisms, such as moulds, may raise the pH of an acid food enough to allow growth of *C. botulinum* spores (Huhtanen *et al.*, 1976; Odlaug and Pflug, 1978).

Table 1. Growth characteristics and thermal inactivation values for spores of proteolytic and non-proteolytic *Clostridium botulinum*

	Growth temperature (°C)			Minimum for growth		
Type	Min.	Max.	Optimum	pH	A_w	*D* values (min)
A (p)	10	50	35-40	4.6	0.94	D_{110} = 2.72-2.89
B (p)	10	50	35-40	4.6	0.94	D_{110} = 1.34-1.37
B (np)	3.3	45	28-30	5.0	0.97	$D_{82.2}$ = 1.49-73.61 $^1D_{85}$ = 100
E (np)	4.0	45	28-30	5.0	0.97	D_{80} = 0.8 $^1D_{91}$ = 13.5
F (p)	10	50	35-40	4.8	0.94	D_{110} = 1.45-1.82
F (np)	3.3	45	28-30	5.0	0.97	$D_{82.2}$ = 0.25-0.84

p = proteolytic
np = non-proteolytic
[1] denotes recovery in the presence of lysozyme

Water activity

Water activity (A_w) is one of the most important controls for *C. botulinum* that is used in foods and is commonly adjusted with NaCl or other humectants such as glucose or sucrose. The limiting A_w for growth of *C. botulinum* is 0.94 for proteolytic types and 0.97 for non-proteolytics, but is also dependent on the solute used. The amount of NaCl required to inhibit growth can be reduced with added nitrite and/or reduced pH.

Temperature

Proteolytic strains will not grow at or below 10°C (Smelt and Haas, 1978), and the lowest temperature recorded for growth of non-proteolytic strains is 3.3°C (Eklund et al, 1967). These experiments are usually carried out in otherwise optimum conditions for growth and the limits will be affected by other factors which may be present in a food.

Preservatives

There are a wide range of preservatives which have been looked at for anti-botulinal activity and the majority have shown some effect to a greater or lesser extent. These include nitrite, which is commonly used in cured products, polyphosphates, ascorbates, sodium metabisulphite, sodium hypophosphite, sorbate, spices, antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, and propyl gallate. A summary of the inhibitory concentrations of these compounds is provided by Rhodehamel *et al.* (1992).

Combined effects

Studies which have looked at the combined effects of a number of factors such as temperature, pH and A_w on growth and/or toxin production (Baird-Parker and Freame, 1967; Ohye and Christian, 1967) provide very useful information for the food processor. Data derived from such experiments has been used to modify formulations and conditions for storage, and also to advise on shelf-life. Recent advances in mathematical modelling techniques have allowed food microbiologists to develop powerful tools which can describe complex interactions between multiple factors. Probabilistic models have been described which can predict the probability of toxin production (Roberts *et al.*, 1981) and kinetic models have been published which predict lag time of toxin production (Dodds, 1989) and growth rates (Baird-Parker and Kilsby, 1987; Whiting and Call, 1993).

Conclusion

The continuing consumer demands for less heavily preserved and/or processed foods inevitably results in an increased likelihood that pathogens, such as *C. botulinum*, will be able to survive and grow in product. Some of these new generation foods are minimally processed, do not contain preservatives and rely solely on refrigeration for safety. Temperature abuse during distribution, retail storage or in the home can, therefore, result in toxin production and foodborne illness. Detailed knowledge of preservation systems and how different factors interact can reduce the likelihood of foodborne illness occurring. Serious consequences can result from mistakes made in formulations or processing, such as the botulism outbreak in hazelnut yoghurt (O'Mahoney *et al.*, 1990). To reduce the calorie content, sugar in the hazelnut purée was replaced with aspartame, resulting in a higher A_w, allowing *C. botulinum* to grow.
Through the use of tools such as predictive models and a good understanding of preservation and processing conditions, identification of critical control points in a process is made much easier. Application of the Hazard Analysis Critical Control Point (HACCP) procedure is now widely accepted as an essential means of assuring food safety and ensuring that foods are 'right by design'.

References

Baird-Parker AC and Freame B (1967) Combined effect of water activity, pH and temperature on the growth of *Clostridium botulinum* from spore inocula. *J. Appl. Bacteriol.*, **30**, 420-429.

Baird-Parker AC and Kilsby DC (1987) Principles of predictive food microbiology. *Soc. Appl. Bacteriol. Symp. Series*, **16**, 43S-49S.

Cerf O (1977) Tailing of survival curves of bacterial spores. *J. Appl. Bacteriol.* **42**,1-19.

Dodds KL (1989) Combined effect of water activity and pH on inhibition of toxin production by *Clostridium botulinum* in cooked, vacuum packed potatoes. *Appl. Env. Microbiol.* **55**, 656-660.

Eklund T, Wieler DI and Poysky FT (1967) Growth and toxin production of non-proteolytic type B *Clostridium botulinum* at 3.3 to 5.6°C. *J. Bacteriol.* **93**, 1461-1462.

Esty JR and Meyer KF (1922) The heat resistance of the spores of *Bacillus botulinus* and allied anaerobes. XI. *J. Infect. Dis.*, **31**, 650-663.

Huhtanen CN, Naghski J *et al.* (1976) Growth and toxin production by *Clostridium botulinum* in moldy tomato juice. *Appl. Env. Microbiol.* **32**, 711-715

Kim J and Foegeding PM (1992) In Hauschild, AHW and Dodds, KL (eds) : *Clostridium botulinum: ecology and control in foods.* Marcel Dekker, 121-176.

M°Clure PJ, Cole MB and Smelt JPPM (1994) Effects of water activity and pH on growth of *Clostridium botulinum*. *J. Appl. Bacteriol. Sympos. Suppl.* 105S-114S.

Odlaug TE and Pflug IJ (1978) *Clostridium botulinum* and acid foods. *J. Food Protec.* **41**, 566-573.

Ohye DF and Christian JHB (1967) Combined effects of temperature, pH and water activity on growth and toxin production by *Clostridium botulinum* types A, B, and E. In Ingram, M and

Roberts TA (eds) : *Botulism*. London, Chapman and Hall, pp 217-223.

O'Mahoney M, Mitchell E *et al.* (1990) An outbreak of foodborne botulism associated with contaminated hazelnut yoghurt. *Epidemiol. Infect.* **104**, 389-395.

Peck MW and Fernandez PS (1995) Effect of lysozyme concentration, heating at 90°C, and then incubation at chilled temperatures on growth from spores of non-proteolytic *Clostridium botulinum*. *Lett. Appl. Microbiol.* **21**, 50-54.

Rhodehamel JE, Reddy RN and Pierson, MD (1992) Botulism: the causative agent and its control in foods. *Food* Control, **3**, 125-143.

Roberts TA, Gibson AM and Robinson A (1981) Prediction of toxin production by *Clostridium botulinum* in pasteurized pork slurry. *J. Food Technol.* **16**, 337-355.

Smelt JPPM and Haas H (1978) Behaviour of proteolytic *Clostridium botulinum* types A and B near the lowest temperature limits for growth. *Euro. J. Appl. Microbiol. Biotechnol.* **5**, 143-154.

Smelt JPPM, Raatjes GJM *et al.* (1982) Growth and toxin formation by *clostridium botulinum* at low pH values. *J. Appl. Bacteriol.* **52**, 75-82.

Tanaka N (1982) Toxin production by *Clostridium botulinum* in media at pH lower than 4.6. *J. Food Protec.* **45**, 234-237.

Townsend CT, Yee L and Mercer WA (1954) Inhibition of the growth of *Clostridium botulinum* by acidification. *Food Res.*,**19**, 536-542.

Van Ermengem E (1897) Ueber einen neuen anaëroben Bacillus und seine Beziehungen zum Botulismus. Zeitschr. f. Hygiene, XXVI, 1-53.

Whiting RC and Call JE (1993) Time of growth model for proteolytic *Clostridium botulinum*. *Food Microbiol.* **10**, 295-301.

Wong DM, Young-Perkins KE and Merson RL (1988) Factors influencing *Clostridium botulinum* spore germination, outgrowth, and toxin formation in acidified media. *Appl. Env. Microbiol.* **54**, 1446-1450.

Clinical aspects of human botulism

EMR Critchley, DM FRCP

Professor of Neurology, Department of Neurology, Royal Preston Hospital, Preston, Lancs., PR2 4HT, UK.

James Lovelock, in Gaia – A New Look at Life on Earth (1990) states that when life began it was anaerobic. When the atmosphere became poisoned by oxygen, anaerobic organisms took refuge in the humus and in the guts of the new aerobic creatures. However, from time to time they take their vengeance on the second generation of living organisms. Toxins from *Clostridium botulinum* are responsible for the deaths of hundreds, if not thousands, of wild, domesticated and exotic species of bird and animal each year and from time to time are a source of mortality and morbidity to humans.

Since human botulism is relatively rare, many of the gaps in our knowledge of the clinical condition can be filled by study of botulism in animals. The first intimation of an epidemic may be deaths of animals or birds for no apparent reason. This does not appear to happen in human foodborne epidemics but there is just a possibility that it may be the explanation for some sudden infant deaths. Birds affected by botulism are characteristically seen with their eyes closed, neck and wings outstretched and legs tucked beneath them. Affected animals initially appear dull and depressed, reluctant to move, stumbling, tremulous and reluctant to stand, reluctant to take food, dribbling or dropping food or unable to swallow, with abdominal distension and low grade colic. Breathing may be laboured and diaphragmatic, the hind limbs splayed and later the forequarters and head assume an abnormal posture, the eyes closed, pupils dilated, tail floppy and the tongue may be grasped and pulled from the mouth. But there are differences between species. Predators who may eat carrion are least affected. In the NW England outbreak a carton of hazelnut yoghurt shared among two children and a cat did not affect the cat. In a zoo outbreak, infected carcasses did not affect coatis or jaguars and caused only mild ataxia but no respiratory paralysis in lions (Greenwood, 1985). Cattle and sheep are less susceptible than horses (Ricketts *et al.*, 1984) and what may kill a horse may not kill a mouse. Few animals ever develop antibodies to botulinum toxins but vaccination has been used to protect cattle, horses, mink and exotic animals in zoos.

Correspondence: Professor EMR Critchley, Department of Neurology, Royal Preston Hospital, Preston, Lancs., PR2 4HT. Tel: 01772 710556; Fax: 01772 718746.

Human botulism is usually an infrequent, sporadic disease and the diagnosis requires a high index of suspicion (Swift and Rivner, 1987). A correct initial diagnosis is more likely once clustering of cases has occurred and a small group of people, usually a family unit or dining companions, can be identified. From the first investigated outbreak in the Belgium village of Ellezelles (von Ermengem, 1896) it has been associated with wakes wherein the guests have drunk (Smith *et al.*, 1970) or eaten (Horowitz *et al.*, 1975) unwittingly the same food which caused the initial death. The patients who succumb with the shortest incubation time tend to be the most severely affected and classical electro-diagnostic abnormalities may not be apparent early in disease (Cherington, 1974). Patients presenting around the same time with other diagnoses, eg Guillain-Barr syndrome, Miller-Fisher syndrome or bilateral strokes with diabetes can add to the difficulties; for example one affected patient believed his symptoms had resulted from a recent fight (Critchley *et al.*, 1989).

The incubation period is variable from 6 hours and as long as 8 days (average 12-36 hours) and many patients will have noticed an abnormal taste or appearance of the food. Where the presentation is oculobulbar in type with double vision, droopy eyes, a nasal or slurred speech and difficulty swallowing, the symptomatology is unequivocal but may progress rapidly (Critchley and Mitchell, 1990). The severity of the later respiratory difficulties often reflects that of the ocular symptoms (Terranova *et al.*, 1979). Characteristically there is a descending paralysis with loss of deep tendon reflexes in proportion to the degree of weakness. The motor weakness is always bilateral but not necessarily symmetrical.

Upper motor neurone signs and cranial involvement, eg transient deafness, can occur but mental

functions and sensations are usually well preserved. However, 40% of patients may develop more protean autonomic nervous system involvement which may precede the muscle weakness or even dominate the clinical picture. One third develop abdominal distension or cramps with vomiting, diarrhoea or constipation. Others develop dry, red or watery eyes, blurred vision, difficulty focusing, dizziness, headache or hypotension. The mouth may be dry, red, hot and infected with coating of the tongue and fauces and strands of stringy saliva, paralytic ileus, urinary retention or regurgitation from the stomach can develop with unexpected rapidity. Patients readily fatigue.

Establishing the diagnosis by serology, or identification of the toxin in stools or food containers can be difficult and may be delayed. Any patient with suspected botulism needs to be hospitalised in the acute stage. Any patient with hypoventilation and oropharyngeal paralysis must be under continuous supervision and repeated observations. The acute onset of bulbar or respiratory signs is always life-threatening. The adage that the time to perform a tracheostomy is when first considered is particularly true of botulism. In well-nourished patients it is more important to protect the airway than to institute nasogastric feeding with the risk of regurgitation into the lungs. Patients with dilation of the stomach, ileus or prolonged swallowing difficulties require feeding parenterally via a central venous line. The risks of secondary infection should also be considered and ventilatory support with other intensive measures may need to be continued for months. Eventually, a near total recovery can be expected in all who survive.

Particularly with types A and E botulism, once the diagnosis can be established early treatment with the appropriate antitoxin is advisable; but late treatment with antitoxin is probably not justified and adds the risk of an anaphylactic reaction to horse serum. Treatment of hypotension with dopamine and antibiotic cover may be required and the electrolytes, cardiograph and urinary output monitored regularly. Guanidine or 4-aminopyridine can be administered to improve peripheral motor function but in the Birmingham outbreak (Ball *et al.*, 1979) failed to improve respiratory function and caused convulsions in one patient. The acute treatment which may need to be continued for weeks or months did not appear to influence the development of chronic symptoms, which are possibly more closely related to the extent of autonomic dysfunction than to the degree of somatic muscular weakness.

Other forms of botulism occur more rarely. Toxico-infective botulism can involve necrotic wounds well described in North America occasionally related to drug abuse (Macdonald *et al.*, 1985). A very clear example is provided by a recently castrated race-horse (Bernard *et al.*, 1987).

In retrospect, the authors' first encounter with botulism was in shaker foal disease – a neuromuscular paralytic disease in foals prevalent in Central Kentucky. Two colleagues of mine in the Department of Neurology, McQuillen and Cantor (unpublished observations), confirmed the presynaptic nature of the neuromuscular block in 1967 but only later was the diagnosis of botulism established (Swerczek, 1980). The disease commonly involved foals between 2 and 4 weeks after periods of stress to lactating mothers as the result of which the fat rich milk contained an excess of corticosteroids causing gastric ulceration. With weaning, spores from contaminated soil and faecal material could be ingested, lodging and proliferating in the necrotic gastric ulcers thus simulating wound botulism.

References

Ball AP, Hopkinson RS, Farrell ID *et al* (1979) Human botulism caused by *Clostridium* botulism type E: the Birmingham outbreak *Q. J. Med.* **48** 473-91.

Bernard W, Divers TJ, Whitlock RH *et al.* Botulism as a sequel to open castration in a horse. *J. Am. Vet. Med. Assoc.* **191** 73-4.

Cherington M (1974) Botulism: ten year experience. *Arch. Neurol.* **30** 432-7.

Critchley EMR, Hayes PJ, Isaacs PET (1989) Outbreak of botulism in North West England and Wales. *Lancet* **ii** 849-53.

Critchley EMR, Mitchell JD (1990) Human Botulism. *Brit. J. Hosp. Med.* 43 290-2

Greenwod AG (1985) Diagnosis and treatment of botulism in lions. *Vet. Rec.***117** 58-60.

Horowitz MA, Marr JS, Merson MH (1975) A continuing common source outbreak of botulism in a family *Lancet* **ii** 861-3

Lovelock J (1990) Gaia - A New Look at Life on Earth. Oxford University Press. Oxford.

Macdonald KL, Rutherford GW, *et al* (1985) Botulism and botulism like illness in chronic drug abuse *Ann. Int. Med.* **102** 616-8.

Ricketts SW, Greet TRC, Glyn PJ *et al* (1984) Thirteen cases of botulism in horses fed big bale silage. *Equine Vet. J.* 16 515-8

Smith DH, Timms GL, Refai M (1970) Outbreak of botulism in Kenyan nomads. *Ann. Trop. Med. Parasit.* **73** 145-8.

Swerczek TW (1980) Toxicoinfective botulism in foals and adult horses. *J. Vet. Med. Assoc.* **178** 217-20.

Swift TR, Rivner MH (1987) Infectious disease of nerve. In: Vinken PJ, Bruyn GW, Klawans HI, Matthews WB eds Handbook of Clinical Neurology. Vol 51. Revised series 7. Elsevier. Amsterdam.

Terranova W, Palumbo JN, Brennan JG (1970) Ocular findings in botulism type B. *J.A.M.A.* **241** 475-7

von Ermengem E (1896) Untersuchugen Uber Fulle von fleischer Giftung mit Symptomen von Botulism. *Z. Nakteriol* **19** 442

Intestinal Toxaemia Botulism in Infants and Adults

Stephen S. Arnon, M.D.

Senior Investigator and Chief Infant Botulism Prevention Program, California Department of Health Services, Berkeley, California 94704-1011, USA.

Keywords: botulism, infant botulism, intestinal toxaemia botulism, Botulism Immune Globulin (Human), botulinum toxin, honey, randomised trial, Clostridium botulinum, Clostridium baratii, Clostridium butyricum

Introduction

Just 20 years ago in July 1976, investigations began in California that resulted in the recognition of a third form of human botulism, infant botulism (Pickett et al. 1976), (Midura and Arnon 1976), (Arnon et al. 1977). These investigations demonstrated the novel infectious disease pathogenesis of infant botulism, namely, the intra-intestinal production of botulinum toxin that, after absorption, acted systematically at the neuromuscular junction to produce hypotonia and flaccid paralysis (Figure 1; Wilcke et al. 1980), (Mills and Arnon 1987). In the course of these investigations the record of a laboratory-confirmed but misidentified case of infant botulism that occurred in 1931 was discovered in the archives of the California Department of Health Services, thereby establishing that infant botulism was not a new disease, but simply a newly-recognised one (Arnon et al. 1979). Parenthetically, botulinum toxin exists in seven serotypes, arbitrarily designated by the letters A-G, that are distinguished from each other by the lack of cross-neutralisation; the seven toxin types serve as convenient clinical and epidemiological markers.

In the years that followed 1976, investigation of clinical cases of infant botulism led to the discovery of new and unique strains of *Clostridium baratii* (Hoffman et al. 1982), (McCroskey et al. 1991) and of *Clostridium butyricum* (Aureli et al. 1986), each of which was found to produce a botulinum-like neurotoxin that caused the infant's illness. Recognition of these additional neurotoxigenic clostridia, together with the occurrence in the past decade of exceptionally rare cases of infant-type botulism in adults, made evident the need for a more inclusive term for these patients; accordingly, this unique disease pathophysiology is now referred to as "intestinal toxaemia botulism" (Arnon 1995; Table 1).

This communication presents central aspects of infant botulism, summarises the rare cases of adult intestinal toxaemia botulism, and reports selected interim data available from the Randomised Trial of Human Botulism Immune Globulin (BIG) for the treatment of infant botulism. That study, now almost complete, had just begun at the time of the earlier International Conference on the Clostridial Neurotoxins held in Madison, Wisconsin in May 1992 (Arnon 1993).

Figure 1 Mother and seven-week-old child with early, mild infant botulism. Note Ptosis, expressionless face and lack of neck, arm and truncal muscle tone.

Table 1. Features of infant-type intestinal toxaemia botulism

Caused by spore-forming, anaerobic bacteria (i.e. the clostridia).
Results from ingestion of spores, which germinate and colonize the large (and perhaps small) intestine.
Toxin then produced by vegetative bacterial cells in the gut; toxin crosses the intestine, enters the bloodstream, and is carried to the neuromuscular junction.
Ranges widely in the rapidity or slowness of onset and in the mildness or severity of symptoms.
Occurs rarely: in California about 1 hospitalized case of infant botulism per 10,000 births.
Adults with intestinal toxaemia botulism appear to have underlying alterations of normal intestinal anatomy, physiology and microflora that predisposes them to disease. Changes include surgical alterations, inflammatory bowel disease, and prior broad-spectrum antibiotic treatment.

Pathophysiology and epidemiology of intestinal toxaemia botulism

Questions often arise as to why intestinal toxaemia botulism is almost exclusively a disease of infants, and what circumstances may explain its extraordinarily rare appearance in adults. These questions need to be considered in the context of the ecology of the clostridia, and especially, of *C. botulinum*. *Clostridium botulinum* (Group I in particular) forms hardy, heat-resistant spores that are ubiquitously present worldwide in soils and dusts, both macroscopic and microscopic. As a consequence, its spores may be found in fresh and cooked fruits, vegetables and other unpreserved agricultural products such as honey. Hence, adults and older children regularly ingest as well as inhale on microscopic dust the spores of *C. botulinum*, while infants mainly inhale the spores. Inhaled spores that adhere to oral, nasal and tracheal secretions are then swallowed and even-

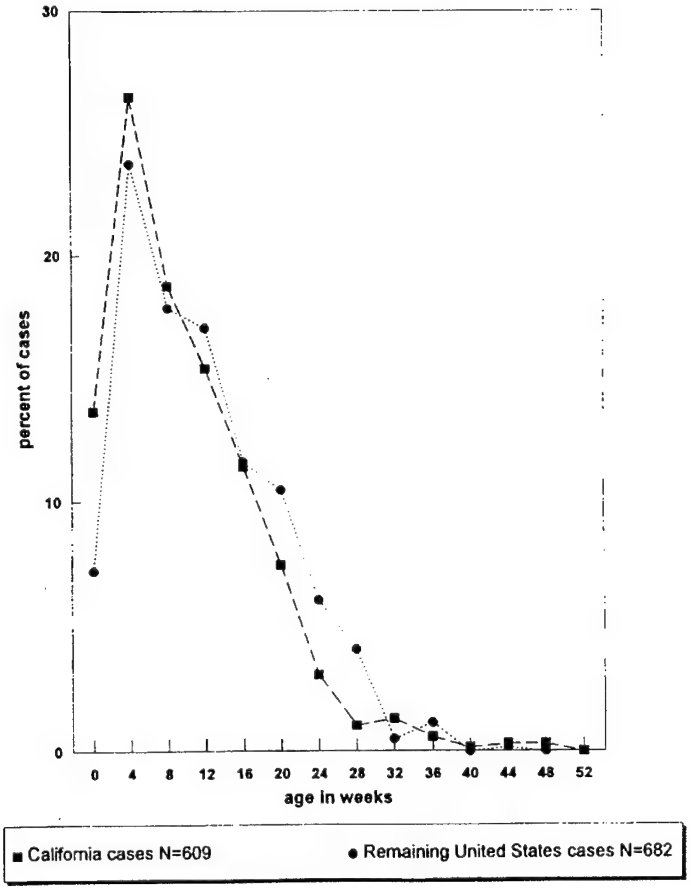


Figure 2. Distribution of age at onset of infant botulism cases, California and the remaining United States, 1976–1994. Onset defined as when parent or care-giver first contacted medical personnel for child's symptoms of Infant botulism.

tually, like ingested spores, reach the large intestine.

Elegant experimental studies in mice by Sugiyama and colleagues (Sugiyama and Mills 1978), (Wells et al. 1982), (Burr et al. 1985), (Sugiyama 1986) (Sugiyama et al. 1986) have demonstrated the central role of the large intestinal bacterial flora in either permitting or inhibiting the germination and outgrowth of *C. botulinum* spores. When the resident intestinal flora is fully developed and diversified, as happens with a stable food diet, the resident flora normally inhibits *C. botulinum* (and other) spore outgrowth, thus explaining how most people can regularly ingest botulinum spores without becoming ill from intestinal toxaemia botulism. In contrast, infants subsist on a predominantly, and often exclusively, milk diet. Hence, their intestinal flora is much simpler, with fewer genera and species (Stark and Lee 1982a, 1982b), and it consequently contains "ecological niches" into which *C. botulinum* can grow. As a result, if an infant has the misfortune to swallow *C. botulinum* spores at a time when his or her flora is permissive to their germination and outgrowth, intestinal colonisation with toxin production by *C. botulinum* may occur.

The age distribution of the proportionate occurrence of infant botulism throughout the first 12 months of life displays the relative frequency of intestinal conditions conducive to *C. botulinum* colonisation, assuming a constant rate of exposure to spores (Figure 2). It is evident that infants are most susceptible to intestinal colonisation and toxin production between two and four months of age, with 60% of cases occurring during this age interval. However, the specific physiological circumstances in the gut that permit spore germination and outgrowth remain unknown. Although some understanding exists of the human infant intestinal conditions and microflora that either permit or inhibit *C. botulinum* colonisation, much remains to be learned (Sullivan et al. 1988).

About a dozen cases are known of intestinal toxaemia botulism in older children and adults. Table 2 displays their pertinent descriptive features. Because ingestion of *C. botulinum* spores among adults and older children is so widespread, the question posed by these extraordinarily rare cases is what initially rendered them susceptible to spore germination and intestinal colonisation. In most cases the answer appears to be changes in the normal gastrointestinal anatomy, physiology and microflora. Normal intestinal anatomy and physiology have been altered by surgery and inflammatory bowel disease, while the microflora has been altered unintentionally by prior antibiotic treatment for unrelated illnesses. The one case that appears to lack predisposing intestinal conditions is the Icelandic man who developed botulism about seven weeks after ingesting food contaminated with *C. botulinum* and botulinum toxin that had earlier made other family members ill with foodborne botulism; he may have developed illness because he ingested a particularly large inoculum of *C. botulinum* spores, or vegetative cells, or both (McCroskey and Hatheway 1988). However, because intestinal surgery, inflammatory bowel disease and antibiotic usage are so common, and intestinal toxaemia botulism in adults is so rare, there are almost certainly other factors that contributed to the development of clinical illness in these dozen or so patients.

Table 2. Confirmed or probable intestinal toxaemia botulism in older children and adults

Patient age/sex, Year	Location	Organism/Toxin Type
1. 37/F 1985	Maryland, USA	*C. botulinum*/A
2. 33/F 1978	Oregon, USA	" /B
3. 45/F 1986	Texas, USA	" /A
4. 70/M 1973	Kentucky, USA	" /B
5. 27/M 1981	Iceland	" /B
6. 54/M 1987	Georgia, USA*	*C. baratii* /F
7. 3/F 1992	California, USA	*C. botulinum*/A
8. 67/M 1988	Oregon, USA	"
9. 48/F 1990	Indiana, USA	" /B
10. 51/F 1990	California, USA	" /B
11. 46/M 1977	California, USA	" /A
12. 62/M 1995	Utah, USA	*C. baratii* /F
13. 54/F 1995	Virginia, USA	" /F
14. 56/M 1995	California, USA	*C. botulinum*/A

* Adapted from Table 10 in Arnon (1995).

Like *C. botulinum* itself, cases of infant botulism have been recognised worldwide and have been reported from all inhabited continents except Africa. The absence of reported cases from Africa probably reflects the generally high infant morbidity there that obscures rare diseases, a possible lack of awareness by physicians, and the scarcity of laboratories capable of performing diagnostic testing. Table 3 summarises laboratory-confirmed cases of infant botulism known as of December 1994.

Risk factors for contracting infant botulism include a slow intestinal transit time (< 1 bowel movement/day, but present in only half the cases) (Spika et al. 1989; Schwarz et al. 1993) and ingestion

Table 3. Gobal recognition of 1420 infant botulism cases 1976-1994[a]

United States	1356	Czech Republic	1
Canada	7	France	1
China	1	Germany	1
Japan	13	Hungary	1
Taiwan	1	Italy	5
Poland	1	Spain	1
Argentina	8	Sweden	1
Chile	1	Switzerland	1
Australia	15	United Kingdom	3
Israel	1	Yemen	1

[a] Data from national surveillance systems or published reports.

of honey, a known reservoir of *C. botulinum* spores. Table 4 displays the 32 known instances in which the actual honey ingested by an infant botulism patient was found to contain *C. botulinum* spores; in every instance the toxin type produced by the spores (A or B) matched the toxin type of the patient's illness. The probability that these 32 consecutive perfect matches occurred solely by chance is less than one in one billion–a strength of association rarely seen in biological systems, and strong evidence that the spores in the ingested honey were the cause of the infant's illness. Corn syrups are no longer considered to be a risk factor for infant botulism (Arnon 1995). In 1996 the United Kingdom became the first governmental jurisdiction to require that retail honey be labelled with a warning statement. The UK label reads simply, "unsuitable for infants under twelve months."

Clinical management and the randomised trial of human botulism immune globulin

Table 5 summarises guidelines for the management of infant botulism patients derived from experience with over 700 hospitalised California patients for the twenty-one year period 1976-1997. Proper positioning of the patient with cervical vertebral support is most important. General patient management is based on two principles, namely, 1) that fatigue as a result of any repetitive muscle activity (e.g., feeding, breathing) is the hallmark of botulism, and 2) that anticipation of complications is the best way to avoid them. Table 6 lists recognised complications of infant botulism.

The randomised trial of human botulism immune globulin (BIG) is a double-masked, placebo-controlled evaluation of the safety and efficacy of BIG for

Table 4. Concordance of toxin type of patients' illnesses and *Clostridium botulinum* spores in their honey

LOCATION	YEAR	TOXIN TYPE IN HONEY	TOXIN TYPE OF INFANT'S ILLNESS	LOCATION	YEAR	TOXIN TYPE IN HONEY	TOXIN TYPE OF INFANT'S ILLNESS
California	1976	B	B	California	1984	B	B
California	1976	B	B	California	1984	B	B
Utah	1978	A	A	California	1984	B	B
California	1977	B	B	California	1984	B	B
Utah	1977	B	B	Canada	1985	A	A
Missouri	1978	B	B	California	1985	B	B
Washington	1978	A	A	California	1985	B	B
California	1979	A	A	Japan	1986	A	A
California	1979	A	A	Japan	1987	A	A
Kentucky	1979	B	B	Japan	1987	A	A
California	1982	A	A	Japan	1987	A	A
California	1983	B	B	Japan	1989	A	A
California	1983	B	B	Italy	1991	B	B
California	1983	B	B	Massachusetts	1994	A	A
California	1984	B	B	New Jersey	1995	A	A
California	1984	B	B	Canada	1995	A	A

Note: This table displays the results of a "forbidden experiment": parents unknowingly fed their babies honeys that were later found to contain *Clostridim botulinum* spores. In every instance, the spore toxin type (A or B) of illness was the same as the toxin type of spore in the honey. The probability of 32 such consecutive instances of a perfect match is less than one in one billion (i.e., $< 10^{-9}$).

Table 5. Principles of management of infant botulism cases

1. POSITIONING:	Crib bed at 30, cloth roll under neck
2. MONITORING:	Transcutaneous 0_2, CO_2; cardiac and respiratory
3. BREATHING:	Protect airway (endotracheal tube); ventilate as needed
4. FEEDING:	Nasogastric or nasojejunal tube feeding; mother's milk is best
5. ELIMINATION:	Bladder credé emptying (no indwelling catheter); use softeners for stool
6. CENTRAL NERVOUS SYSTEM:	No sedatives or hypotonics
7. ANTIBIOTICS:	Only for secondary infections (lung, UTI most common)
8. ANTITOXIN:	Equine derived contraindicated Human derived may be available soon (clinical trial of BIG ends spring 1997)

Table 6. Complications of infant botulism

Adult respiratory distress syndrome
Aspiration
Clostridium difficile colitis
Fracture of the femur (nosocomial)
Inappropriate antidiuretic hormone secretion
Misplaced or plugged endotracheal tube
Necrotizing enterocolitis
Otitis media
Pneumonia
Recurrent atelectasis
Seizures secondary to hyponatremia
Sepsis
Subglottic stenosis
Tension pneumothorax
Tracheal granuloma
Tracheal stenosis
Tracheitis
Tracheomalacia
Transfusion reaction
Urinary tract infection

the treatment of infant botulism (Arnon 1993). It was designed to enrol 120 laboratory-confirmed patients, the number expected to occur in a usual three-year period in California. The primary outcome measure is a significant reduction in the length and cost of hospital stay (Tables 7, 8). BIG was obtained by plasmapheresis of volunteers who had been immunised with botulinum toxoid for occupational safety reasons. The placebo is licensed, commercially-available normal human immune globulin (Gammagard S/D, Baxter). Approximately 90 hospitals represented by 62 Institutional Review Boards are participating in the clinical trial because even in California, the infrequent occurrence of this Orphan Disease (30-40 hospitalised cases/year) necessitates having a large number of catchment hospitals.

Dosage of BIG and placebo is standardised at 50 mg/kg. The titre of neutralising antibody in BIG (25 IU/ml anti-A, 5 IU/ml anti-B) is high enough for a single intravenous infusion to provide protection against botulinum toxaemia for at least six months (Table 9). The study enrolled its first patient in February 1992, and enrolment as of December 31, 1996 stood at 113 laboratory-confirmed cases, or 94% of planned enrolment. The 120th, last patient is projected to be enrolled in February 1997.

Because the study will remain masked until the

Table 7. Randomised trial of human botulism immune globulin (BIG)

THERAPEUTIC PRINCIPLE: Maximise neutralisation of toxaemia by minimising of the interval between disease onset and BIG administration.

STUDY DESIGN: Placebo-controlled, double-masked, randomised. One *iv* dose of BIG (50 mg/kg) is expected to provide >6 months of toxaemia neutralisation.

PLACEBO: Commercially-available, FDA-licensed, normal human IGIV (Gammagard S/D, Baxter).

OUTCOME MEASURES: (1) Length and cost of hospital stay, (2) progression of illness, (3) incidence of complications.

LOGISTICS: Coded vial hand-delivered to bedside in response to telephone enquiry. Five-year enrollment of 120 patients planned. Approximately 90 participating hospitals; 62 separate Institutional Review Board approvals obtained.

COST TO PATIENT OR HOSPITAL: None.

SUPPORT: Orphan Drug Program, U.S. Food and Drug Administration;California Department of Health Services.

Table 8. Clinical trial of human botulism immune globulin (BIG) for the treatment of infant botulism (February 24, 1992 - December 31, 1996).

Patients Enrolled:	121 (49 male, 72 female)
Laboratory-Confirmed Cases with data available:	113 (68 type A, 45 type B)
Length of Hospital Stay:	0.7 - 27.7 weeks
Cost of Hospital Stay:	$5,232 - $579,923
Participating Hospitals:	88 (62 separate IRBs)

Table 9. Duration and titres of botulism immune globulin (big) at a dosage of 50 mg/kg

Day	anti-A, IU/ml x 10^{-4}	anti-B, IU/ml x 10^{-4}
0	1250	250
30	625	125
60 (2 months)	312	63
90	156	32
120	78	16
150	39	8
180 (6 months)	20	4

last patient has been discharged from hospital, the data presently available on outcome measures can only be viewed by comparison of the aggregate study patient experience (treatment and placebo arms combined) and the historical experience accumulated during the 15-year pre-trial period 1976-1991. These data are displayed in Figures 3-5. Inspection of these figures makes evident that during the BIG study period, the aggregate length and cost

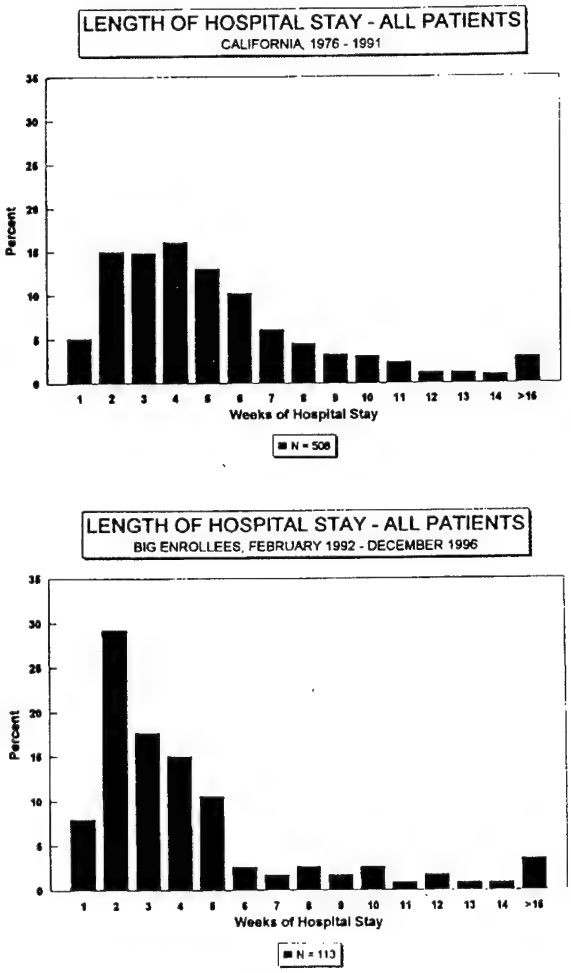


Figure 3 Comparison of length of hospital stay for California infant botulism patients before (upper panel) and during (lower panel) the randomized trial of human Botulism Immune Globulin (BIG).

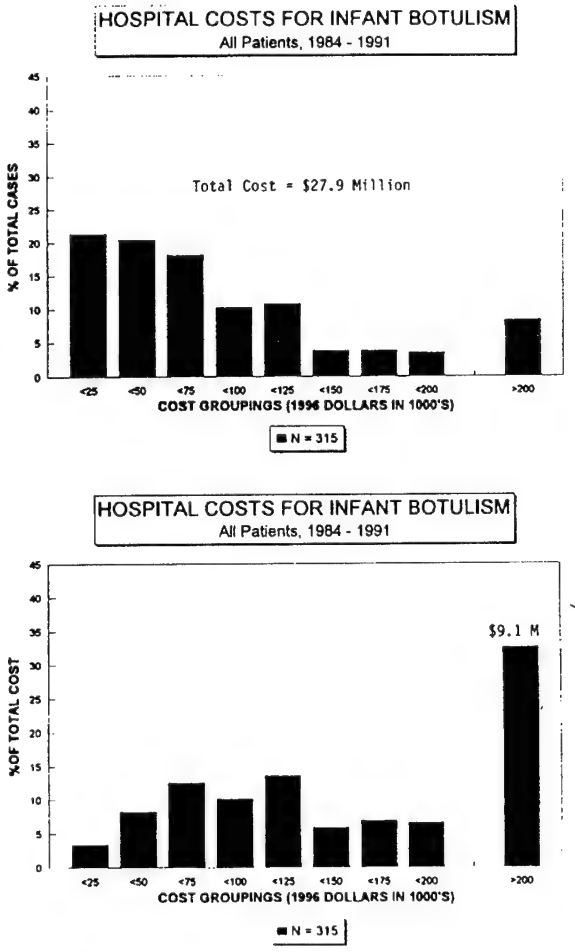


Figure 4 Hospital costs for California infant botulism patients before the randomized trial of human Botulism Immune Globulin (BIG). Upper panel: distributions of cases by cost. Lower panel: proportion of the total costs contributed by each cost grouping.

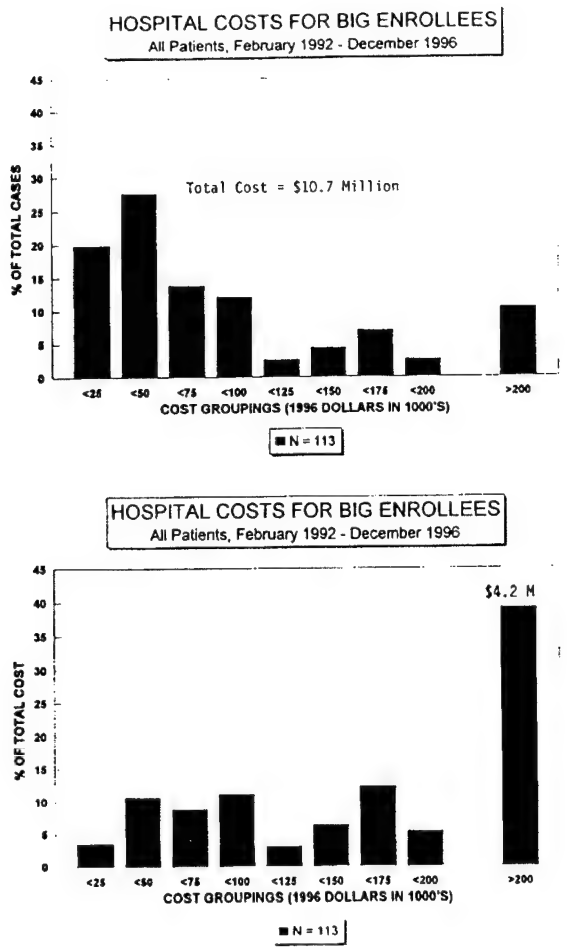


Figure 5 Hospital costs for California infant botulism patients enrolled in the randomized trial of human Botulism Immune Globulin (BIG). Upper panel: distribution of cases by cost. Lower panel: proportion of the total costsa contributed by each cost grouping.

of hospital stay have diminished for the enrolees taken as a group.

Once the last patient has been discharged and the study code has been broken, the full and appropriate data analysis will be performed by directly comparing the experience of the treatment and placebo arms of the study with regard to length and cost of hospital stay. If the results demonstrate safety and efficacy, then a licensure application will be submitted to the US Food and Drug Administration. If a license is issued, then BIG will become, more than 20 years after modern recognition of the disease, the first therapeutic product specifically indicated for, and effective in, the treatment of infant botulism.

References

Arnon SS (1993) Clinical Trial of Human Botulism Immune Globulin. In DasGupta BR (ed): *Botulism and Tetanus Neurotoxins: Neurotransmission and Biological Aspects*. New York: Plenum Press, 477–82.

Arnon SS (1995) Botulism as an Intestinal Toxaemia. In Blaser MJ, Smith PD, Ravdin JI, Greenberg HB, and Guerrant RL (eds): *Infections of the Gastrointestinal Tract*. New York: Raven Press, 257–71.

Arnon SS, Midura TF, Clay SA, Wood RM, Chin J (1977) Infant botulism: epidemiological, clinical, and laboratory aspects *The Journal of the American Medical Association* **237,** 1946–51.

Arnon SS, Werner SB, Faber HK, Farr WH (1979) Infant botulism in 1931: discovery of a misclassified case *Am J Dis Child* **133,** 580–582.

Aureli P, Fenicia L, Pasolini B, Giafranceschi M, McCroskey LM, Hatheway C. L. (1986) Two cases of type E infant botulism in Italy caused by neurotoxigenic *Clostridium butyricum J Infect Dis* **23,** 201–2.

Burr DH, Beery JT, Sugiyama H (1985) Importance of cecum in *Clostridium botulinum* colonization of mice and relationship of organism to large bowel *Current Microbiology* **12,** 277–82.

Hoffman RE, Pincomb BJ, Skeels MR, Burkhart MJ (1982) Type F infant botulism *Am J Dis Child* **136,** 270–1.

McCroskey LM, Hatheway CL (1988) Laboratory findings in four cases of adult botulism suggest colonization of the intestinal tract *J Clin Microbiol* **26,** 1052–4.

McCroskey LM, Hatheway CL, Woodruff BA, Greenberg JA, Jurgenson P (1991) Type F botulism due to neurotoxigenic *Clostridium botulinum* from an unknown source in an adult *J Clin Microbiol* **29,** 2618–20.

Midura TF, Arnon SS (1976) Infant botulism: identification of *Clostridium botulinum* in faeces *Lancet* **2,** 934–936.

Mills DC, Arnon SS (1987) The large intestine as the site of *Clostridium botulinum* colonization in human infant botulism *J Infect Dis* **156,** 997–8.

Pickett J, Berg B, Chaplin E, Brunstetter-Shafer M (1976) Syndrome of botulism in infancy: clinical and electrophysiologic study *N Engl J Med* **295,** 770–2. Schwarz PJ, Arnon, JM, Arnon SS (1993) Epidemiological aspects of infant botulism in California. In DasGupta BR (ed): *Botulism and Tetanus Neurotransmission and Biological Aspects*. New York: Plenum Press, 477–82.

Spika JS, Shaffer N, Hargrett-Bean N, Collin S, MacDonald KL, Blake PA (1989) Risk factors for infant botulism in the United States *Am J Dis Child* **143,** 828–32.

Stark PL, Lee A (1982a) The microbial ecology of the large bowel of breast-fed and formula-fed infants during the first year of life *J Med Microbiol* **15,** 189–203.

Stark PL, Lee A (1982b) Clostridia isolated from the feces of infants during the first year of life *J Pediatr* **100,** 362–5.

Sugiyama H (1986) Mouse models for infant botulism *Exp Mdls Antimicrob Chemother* **2**: 73–88.

Sugiyama H, Mills DC (1978) Intraintestinal toxin in infant mice challenged intragastrically with *Clostridium botulinum* spores *Infect Immun* **21,** 59–63.

Sugiyama H, Woller MJ, Prather J (1986) Intestinal colonization of mice by air-disseminated *Clostridium botulinum Current Microbiology* **13,** 85–9.

Sullivan NM, Mills DC, Riemann HP, Arnon SS (1988) Inhibition of growth of *Clostridium botulinum* by intestinal microflora isolated from healthy infants *Microbiol Ecol Hlth Dis* **1,** 179–92.

Wells CL, Sugiyama H, Bland SE (1982) Resistance of mice with limited intestinal flora to enteric colonization by *Clostridium botulinum J Infect Dis* **146,**6 :791–796.

Wilcke BW Jr, Midura TF, Arnon SS (1980) Quantitative evidence of intestinal colonization by *Clostridium botulinum* in four cases of infant botulism *J Infect Dis* **141,** 419–23.

Improved vaccines and treatments of tetanus on the basis of the structure and function of the tetanus molecule to reduce tetanus deaths to zero.

Morihiro Matsuda[1], Motohide Takahashi[2], Dian-Liang Lei[1], Jun Katahira[1] and Nakaba Sugimoto[1]

[1]Department of Bacterial Toxinology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565 and [2]Department of Bacterial and Blood Products, National Institute of Health, 4-7-1 Gakuen, Musashimurayama, Tokyo 208, Japan.

Key words: tetanus, fragment vaccine, human monoclonal antitoxins, blood pressure control

Currently there are three problems associated with the current prophylaxis and treatment of tetanus. These are (1) adverse reactions after the vaccination with current tetanus toxoids, (2) possible risks of immunological and blood-borne infections associated with current tetanus antitoxins and their limited sources, (3) uncontrollable fluctuations of systemic blood pressure which are often observed after controlling hyperactivity of motor systems in severe cases of tetanus. We have addressed these in the following way. Formalin-treated preparations of Fragment [B•C] of tetanus toxin showed protective immunopotency equivalent to whole toxin-toxoid and lesser local reactions than the latter. Sufficient doses of human monoclonal antibodies produced by mouse/human-human hybridomas which had high toxin neutralising activities individually completely protected mice against toxin. A combination of two monoclonal antibodies, which recognize Fragment [B] and [C] of the tetanus toxin molecule respectively, had a potency that was almost equivalent to the human poloyclonal antitoxin currently used. We developed animal models showing remarkable fluctuations of blood pressure following injection of tetanus toxin. Using these animal models, methods for controlling the fluctuation of blood pressure by continuous total spinal anesthesia were developed. The improvements to tetanus vaccines and treatments outlined here may significantly reduce the number of deaths due to this toxin.

Introduction

To reduce tetanus deaths to zero is one of the most important goals in the study on tetanus toxin. According to the statistics of the World Health Organisation, we still have ca. one million deaths of tetanus every year in the world although tetanus is one of the diseases that can be prevented by active imunization. For more than one hundred years since the discovery of antitoxin therapy for the disease by Behring and Kitasato, horse antitoxin preparations and more recently anti-tetanus IgG preparations have been used worldwide for the treatment of tetanus. The problems encountered currently with tetanus vaccines and treatments of tetanus are (1) adverse local and /or general reactions following active immunization, (2) limited sources of human anti-tetanus IgG preparations and possible risks of viral infections associated with the use of human serum preparation, and (3) uncontrollable fluctuations of blood pressure due to disturbance of the autonomous nervous system which is often observed in severe cases of tetanus after controlling hyperactivity of motor systems in ICU. In order to reduce the number of tetanus death, it is necessary to solve these problems, in addition to promoting an expanding vaccine immunisation program.

Correspondent author: Morihiro Matsuda, M.D., Department of Bacterial Toxinology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565, Japan
Telephone: 06-879-8284; Telefax: 06-879-8283

An improved tetanus vaccine composed of the heavy chain of the tetanus toxin molecule

As listed in Table 1, current tetanus vaccines composed of partially purified tetanus toxin-toxoid

Table 1. Side Reactions Following Tetanus Immunization

Local Reactions:
Bullous Inflammation, Rash, Erythema, Macula
Pruritus, Nodule
Edema, Necrosis
Generalized Reactions:
Headache, Fever, Malaise
Lymphopathy, Paresthesia, Paresis, Paralysis
Urticaria, Pruritus, Arthralgia, Arthritis
Anxiety, Tremor, Dizziness, Vertigo, Vomiting
Dyspnea, Anoxia, Hypertona, Tachycardia
Convulsions, Syncope
Anaphylactoid, Anaphylactic Shock
Death

Data Obtained from World Health Organization

elicit adverse reactions, though generalised adverse reactions following immunization with tetanus vaccine are relatively rare. Local reactions at the site of vaccine injections, such as erythema, apaule and induration, are more frequently observed. To minimize these adverse reactions, we tried to develop a toxin fragment preparation having a similar degree of antitoxic immunopotency to the current tetanus whole toxin-toxoid but with limited antigenic sites, i.e. a fragment vaccine. The tetanus toxin molecule is composed of three functional domains (Fig 1). We have previously established the methods of purification for the toxin fragments which correspond to each domain together with a complex of the two adjacent domains (Matsuda and Yoneda, 1975, 1976; Ozutsumi et al., 1989; Matsuda et al., 1989). To explore which of the fragments had a high protective immunopotency, we immunized groups of mice (n=10) with various amounts of formalin-treated

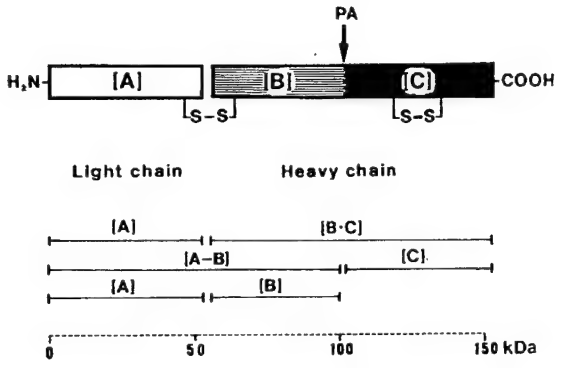


Fig. 1. A schematic diagram representing the structure of tetanus toxin and its fragments

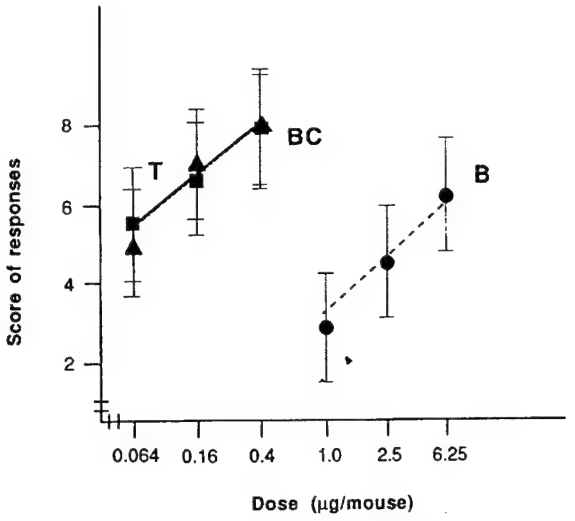


Fig. 2. Comparison of protective immunopotencies of preparations derived from whole toxin (T), Fragment [BC] (BC), and Fragment [B] (B). Mice were immunized with each preparation, and 4 weeks later challenged by subcutaneous injection of 100 LD_{50} of tetanus toxin. Deaths and symptoms of mice were recorded for one week and the results converted to scores by the method of Kameyama and Kondo (1975).

fragment preparations derived from each purified fragment or their combinations. Four weeks later, the mice were challenged by subcutaneous injection of 100 LD_{50} of tetanus toxin, and the mice observed for one week for symptoms and/or death. The results on protective activity were converted to scores by the method of Kamevama and Kondo (1975) with a slight modification to estimate the relative immunopotencies of the fragment preparations to that of whole toxin-toxoid (Fig.2). The results are summarized in Table 2. Among the fragment preparations, those derived from Fragment [BC] gave the highest amount of protection comparable to that of the

Table 2. Relative Immunopotency of Tetanus Toxin Fragments and their Mixtures Compared to Whole Toxin-Toxoid

Antigen	Potency	(95% Confidence Interval)
Toxin-Toxoid	**1.0**	
[A] (L)	0.133	(0.065 ~ 0.272)
[B] (H_N)	0.020	(0.010 ~ 0.042)
[C] (H_C)	0.311	(0.151 ~ 0.644)
[A-B]	0.221	(0.108 ~ 0.453)
[B•C]	**1.158**	(0.563 ~ 2.382)
[A]+[C]	0.333	(0.159 ~ 0.698)
[B]+[C]	0.245	(0.114 ~ 0.529)
[A]+[B]+[C]	0.329	(0.160 ~ 0.675)

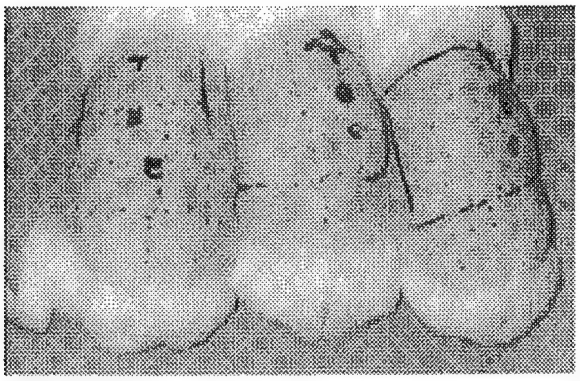


Fig 3. Local skin reactions observed at the sites of intradermal injection of antigen preparations. Guinea pigs were each sensitized by subcutaneous injection with 10μg of aluminium hydroxide-adsorbed preparation derived from Fragment [BC] (B), from purified whole toxin-toxoid (T), or commercially available tetanus toxoid (C). Four weeks later, the antigen preparations at various doses (32, 10 and 3.2μg) were injected intradermally into the guinea pigs.

whole toxin-toxoid. Indication of skin reactions by this preparation (B preparation) was compared to those caused by a commercially available tetanus-toxoid (C preparation) and a whole toxin-toxoid (T preparation) prepared from purified toxin. Guinea pigs were initially sensitized by subcutaneous injection with 10μg of B, C or T preparations, each adsorbed to aluminium hydroxide, and then B, C, T preparations of 32μg, 10μg and 3.2μg doses were injected (0.1ml) intradermally into the guinea pigs. Figure 3 shows an example of the skin reactions observed in the guinea pigs. These were erythematous papules with (only in the case of pre-sensitization with the C preparation) or without necrosis at the top. The reactions reached a maximum approximately 48h after the intradermal injections and lasted over a week. The skin reactions induced by intradermal injection of B preparation were the smallest in size and weakest in intensity, irrespective of the antigen preparations used in the pre-sensitization of the animals. The toxoid preparation composed of formalin-treated purified toxoid showed lesser skin reactions than those induced by id injection of commercial toxoid preparation. Therefore we conclude that Fragment [B•C] of tetanus toxin may be a useful replacement vaccine for the current tetanus toxoid vaccine since it has a similar degree of antitoxic immunopotency but causes fewer adverse skin reactions.

Human anti-tetanus monoclonal antibodies possessing high neutralizing activities

Initially anti-tetanus sera from horses and then more recently anti-tetanus immunoglobulin preparations from human donors have both been used for the prevention and therapy of tetanus since the discovery of antitoxin therapy. However, horse antitoxin may cause potential allergic complications, whilst the use of human IgG preparations may result in blood-borne infections by viruses and/or unknown transmissible agents. Recent advances in cellular engineering techniques have provided a way to avoid these potential risks. We have established five mouse/human-human hybrid cell lines which produce human monoclonal antibodies (MAbs) that possess high toxin-neutralizing properties and would require minimum processing to prepare them for human use (Kamei et al., 1990; Matsuda et al, 1992). Figure 4 shows an example of the results obtained from the neutralization experiments in mice by these monoclonal antibodies. Interestingly, with doses of MAb below that sufficient to neutralize the toxin, animals

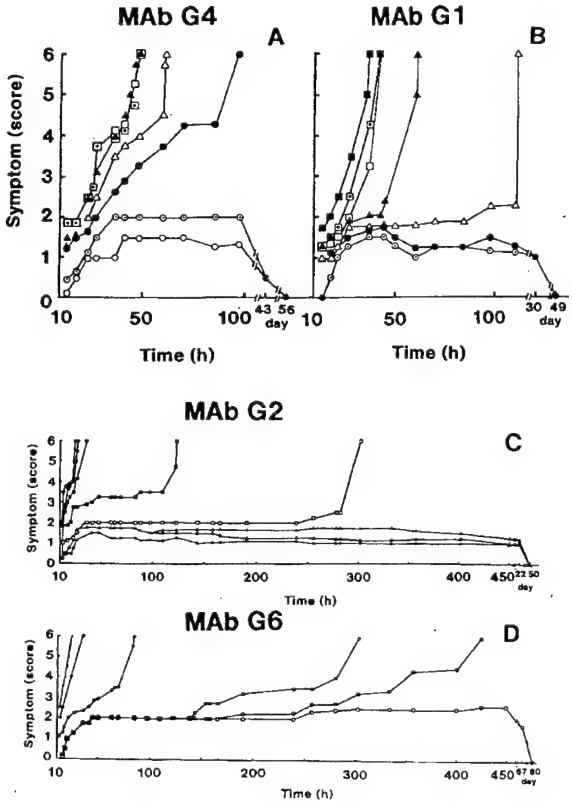


Fig. 4. Neutralization patterns of human monoclonal antibodies. Each monoclonal antibody was incubated with tetanus toxin (20 MLD) at 37C for 1h and the reaction mixtures injected intramuscularly into the hind legs of mice. Tetanus symptoms in the mice were observed and converted to a score by modification of the method of Matsuda et al. (1992).

Table 3. Neutralising Activities of Human Anti-Tetanus Monoclonal Antibodies

Antibody	Domain	Affinity[1] (Ka) ($\times 10^{10}$ M^{-1})	Minimum Survival Dose[2] (MSD) (μg IgG)	IU[3] Per 70μg IgG
G2	[C] (H_C)	11	0.89	1
G4	[A] (L)	14	28	0.03
G6	[B] (H_N)	10	0.28	3
Mixture [4]				
[G2, G4]	[C], [A]		0.28	
[G2, G6	[C], [B]		0.028	
[G4, G6]	[A], [B]		0.89	
[G2, G4, G6]	[C], [A], [B]			30

1. Ka was estimated by ELISA and Scratchcard plot analysis
2. Minimum survival dose (MSD) against 20 MLD of toxin
3. Tetanobulin (human polyclonal IgG antitoxin of known IU) used as a standard
4. Equal amounts of each MAb.

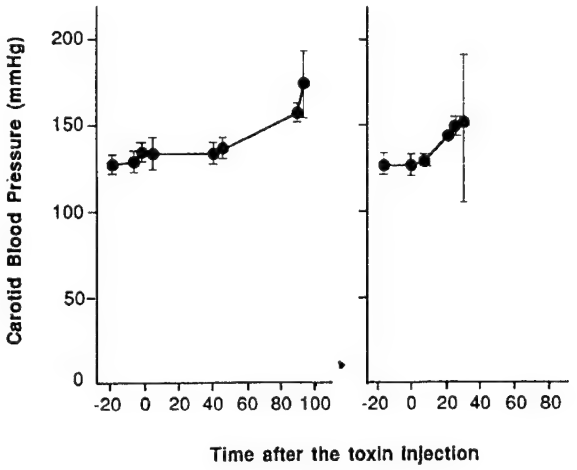


Fig. 5. Mean carotid arterial blood pressure of rats before and after the injection of purified tetanus toxin into a lateral cerbral ventricle. Left column, 2.6ng of tetanus toxin: right column; 26ng of the toxin.

sometimes showed delayed death (in some cases about 3 weeks after the injection of MAb-treated tetanus toxin), although the MAbs both suppressed the development of tetanus symptoms and slowed the rate of progress of the symptoms. Therefore, we determined the minimum dose of MAbs against 20 MLD of toxin to ensure survival of the animals in a comparison of the neutralizing activities of MAbs with that of the human polyclonal antitoxin currently used. Table 3 summarizes the neutralising activities of MAbs designated G2, G4, G6 together with mixtures of antibodies used in combination. The combination of G2 and G6 showed the highest neutralizing activity, comparable to that of the human polyclonal antitoxin currently used. Recently we have succeeded in cloning and sequencing the DNA encoding the variable regions of some of these MAbs to provide improved tetanus antitoxin preparations in the future. These human MAbs are expected to be useful and more ideal tetanus antitoxins than the current preparations.

An animal model of tetanus-induced circulatory disturbance and development of a novel treatment ~~for it~~

The mortality rate of tetanus varies with the severity of the disease, the age of the patients, medical facilities where the patients are hospitalized and between

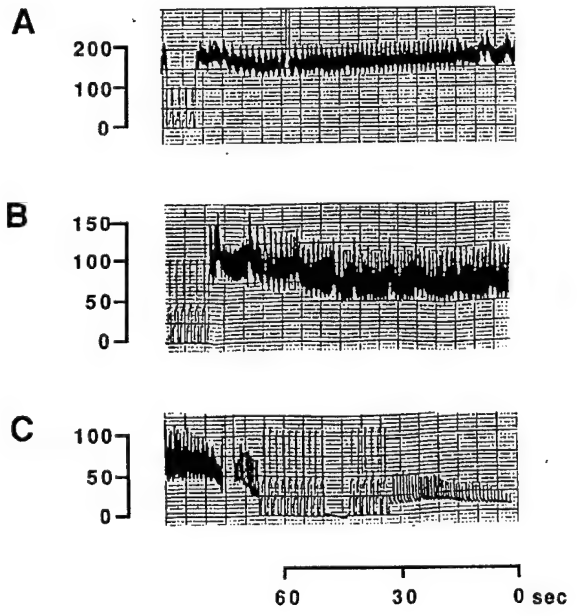


Fig. 6. An example of the record of rat carotid arterial pressure. Purified tetanus toxin (12μg was injected into a lateral cerebral ventricle of a rat. The right carotid artery of the rat was cannulated and the blood pressure monitored using a pressure transducer (TP-400T, Nihonkohden, Tokyo) and an amplifier (AP-601G, Nihonkohden, Tokyo). The upper trace; 5.75h after the injection: the middle trace; 6.4h: the lower trace; 6.6h, respectively.

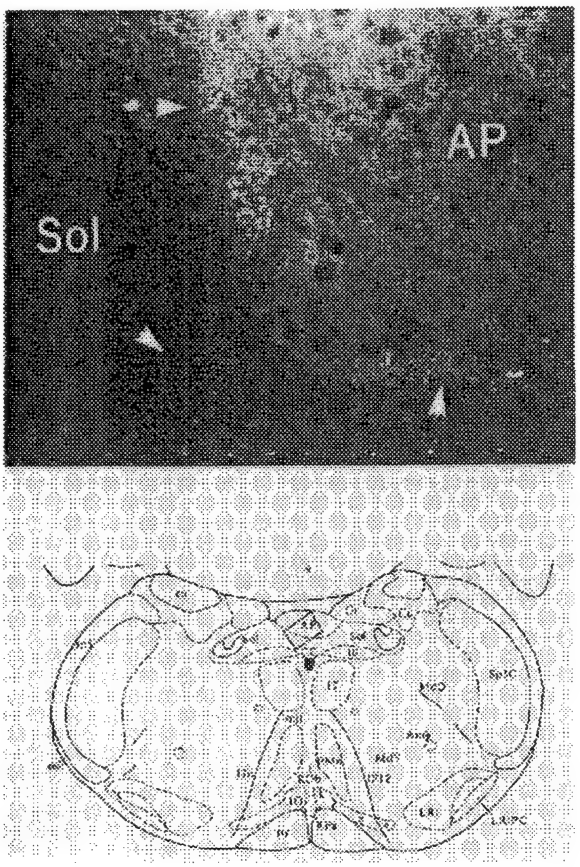


Fig. 7. Immunohistochemistry of the medulla oblongata of a rat in which remarkable fluctuations in blood pressure were observed after injection of tetanus toxin into the lateral cerebral ventricle. A frozen section of medulla oblongata of the rat was stained with fluorescent labelled anti-tetanus antibody. Note the intensive fluorescence at the border of area postrema and solitarius nucleus.

countries. Our University Hospital admits patients with relatively severe tetanus and the fatality rate during 1970-80's was about 50% which is similar to that reported by the Centers for Disease Control at Atlanta in the USA (1987). In severe cases of tetanus, patients often show disturbance in the function of the autonomic nervous system such as large fluctuations of the systemic blood pressure which are difficult to control, despite successful management of the motor systems by curarization and artificial ventilation in ICU. Such disturbances of the autonomic nervous system can be reproduced in rats and rabbits by intracerebroventricular injection or repeated intravenous injections of purified tetanus toxin. Figure 5 and 6 show an elevation of systemic blood pressure and its fluctuation caused by intracerebroventricular injection of the toxin in rats. In these animals, tetanus toxin was detected by immunohistochemical staining at the border of the area postrema and solitarius nucleus in the medulla oblongata (Fig. 7). These results suggest that tetanus toxin directly affects the medullary cardiovascular center. On the basis of these findings the Department of Traumatology and Emergency Medicine of Osaka University Hospital has developed a novel method for controlling the blood pressure in tetanus patients under continuous total spinal anesehesia using bepivacaine (10-25 mg/h) together with epinephrine infusion (90 ng/min, Kg). Figure 8 shows one of the results of this novel treatment. Since then, in contrast to before the introduction of this treatment, all of the tetanus patients admitted to the University Hospital have recovered completely.

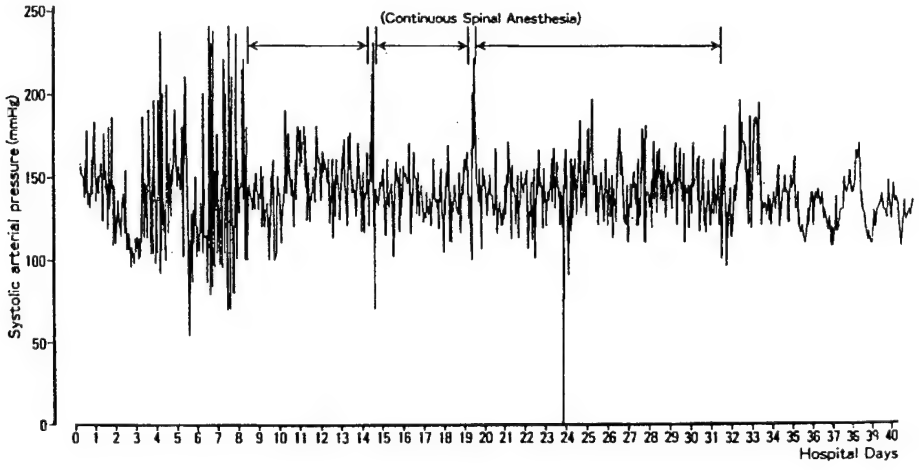


Fig. 8. A clinical record of a case in which continuous total spinal anesthesia was introduced. Note the disappearance of blood pressure after the introduction of continuous total spinal anesthesia with epinephrine infusion on hospital day 8.

Conclusion

Here, we report how we have addressed the current problems associated with methods of prophlyaxis and treatment of tetanus. The fragment vaccine we report here will minimize those adverse reactions normally experienced following tetanus vaccination. The human anti-tetanus monoclonal antibodies we have prepared will be useful in avoiding possible risks of immunological and blood-borne complications associated with current tetanus antitoxins supply of which is limited. Continuous total spinal anesthesia provides a way to overcome the disturbances in the autonomic nervous system associated with tetanus. The causative agent of tetanus has been and always will be a threat to humans since spores of *C. tetani* can be isolated from soil everywhere on the earth. The improved methods of prophylaxis and treatments of tetanus described here could help to reduce significantly the number of deaths due to tetanus.

References

Centres for Disease Control (1987) Tetanus-United States,. 1985-1986. MMWR, 36-477.

Kamei M. Hashizume S. Sugimot N. Ozutsumi K. and Matsuda M. (1990). Establishment of stable mouse/human-human hybrid cell lines producing large amount of anti-tetanus human monoclonal antibodies with high neutralizing activity. Eur J Epidemiol **6**, 386-397.

Kameyama S and Kondo S (1975) Titration of tetanus antitoxin by passive hemagglutination. I. Titration of guinea-pig antitoxins at various periods of immunization. Jpn J Med Sci biol **23**, 127-138

Matsuda M Kamei M Sugimoto N Ma Y and Hashizume S (1992) Characteristics of toxin-neutralization by anti-tetanus human monoclonal antibodies directed against the three functional domains [A], [B], and [C] of the tetanus toxin molecule and a reliable method for evaluating the protective effects of monoclonal antibodies. Eur J Epidemiol **8**, 1-8

Matsuda M Lei D-L Sugimoto N Ozutsumi K and Okabe T (1989) Isolation, purification and characterization of Fragment B, the NH_2-terminal half of the heavy chain of tetanus toxin. Infect Immun **57** 3588-3593.

Matsuda M and Yoneda M (1975) Isolation and purification of two antigenically active, complimentary¤ polypeptide fragments of tetanus neurotoxin. Infect Immun **12**, 1147-1153.

Matsuda M and Yoneda M (1976) Reconstitution of tetanus neurotoxin from two antigenically active polypeptide fragments. Biochem Biophys Res Commun **68** 668-674.

Ozutsumi K Lei D-L Sugimoto N and Matsuda M (1989) Isolation and purification by high performance liquid chromatography of a tetanus toxin fragment (Fragment [A-B] derived from mildly papain-treated toxin. Toxicon **27**, 1055-1057.

Session 2

Genetics

Botulinum neurotoxin complex genes

J. C. Marvaud, M. Gibert and M. R. Popoff*

Unité des Toxines Microbiennes, Institut Pasteur, 28 rue du Dr. Roux, 75724 Paris cedex 15.

Key words: Botulinum neurotoxin, tetanus toxin, *Clostridium botulinum*, electroporation, regulatory gene.

The botulinum neurotoxin (BoNT) complex locus has been identified in different *Clostridium botulinum* serotypes. It consists of 6 genes localized in close vicinity, one coding the BoNT, 5 the associated non toxic proteins (ANTP) and the final gene encodes a basic protein (orf21 in *C. botulinum* A and B, and orf22 in *C. botulinum* C and D) which is related to certain *Clostridium* regulatory proteins. Transformation by electroporation has been developed for genetic study in *C. botulinum*. It was shown that orf21 is a positive regulator of the expression of BoNT and presumably of ANTP genes by using recombinant strains of *C. botulinum* A which overexpressed *orf21* or produced *orf21* antisense mRNA.

Introduction

The botulinum neurotoxin (BoNT) and tetanus toxin (TeNT) share a common structure. They are synthesized as single chain proteins (MW ca. 100 kDa) which are proteolytically cleaved into a light chain (L, MW ca. 50 kDa) and a heavy chain (H, MW ca. 100 kDa) linked by a disulfide bridge. BoNTs act at the neuromuscular junction, blocking the release of neurotransmitter acetylcholine, whereas TeNT prevents the release of the neurotransmitter glycine at inhibitory synapses in the central nervous system. BoNTs and TeNT are responsible for severe neuronal diseases, botulism and tetanus respectively.

BoNTs are divided into seven serotypes (A, B, C1, D, E, F, and G) according to their antigenic properties. They are produced by *Clostridium botulinum* which form an heterogeneous bacterial species and can be divided into four bacteriological groups. Moreover, other *Clostridium* such as some strains of *C. baratii* and *C. butyricum* are able to produce a BoNT type E and F respectively. In contrast, no variation has been found in TeNT and this toxin is produced by only one Clostridium species (*C. tetani*).

In naturally contaminated foods and in culture supernatants, BoNT is associated with non toxic proteins (Associate Non-Toxic Proteins (ANTP) or neurotoxin-binding proteins) by non covalent bonds, thus forming a BoNT complex. The ANTP dissociate from the BoNT above pH 7, and reassociate spontaneously when the pH is lowered. The size of the BoNT complexes vary according to the different BoNT types, from 230 up to 900 kDa. Some of the ANTPs possess hemagglutinating activity (reviewed in Popoff and Eklund, 1995). The biological function of the ANTPs are not well understood. It seems that they are involved in the protection of the BoNT against the acidic pH of the stomach and digestive proteolytic degradation. Therefore, the oral toxicity of BoNT complex is much higher than that of the purified BoNT. In contrast, TeNT does not associate with non toxic proteins to form large complex and it is not active by the oral route.

Here, we present summarized data on the BoNT complex genes, and a number of genetic approaches to investigate the function of the ANTP genes. We also report that *orf21* is a positive regulator of BoNT/A gene and probably of the ANTP genes.

Genetic organization of the BoNT complex

BoNT/C1 complex locus

We have cloned and sequenced the BoNT/C1 gene and its flanking regions from *C. botulinum* C-468 (Hauser *et al.*, 1994). The 12,297 nucleotide fragment encompasses 6 genes which are in close vicinity (less than 62 nucleotides) and constitute the locus of the BoNT/C1 complex (Fig. 1). This locus is separated from the other genes by at least 300 non coding nucleotides in the upstream part and by at least 390 non coding nucleotides in the downstream part.

On the basis of transcription orientation, the 6 genes can be divided into 3 units. The cluster 1

* corresponding author, Phone 33 1 45 68 83 07; Fax 33 1 45 68 84 56
email mpopoff@pasteur.fr

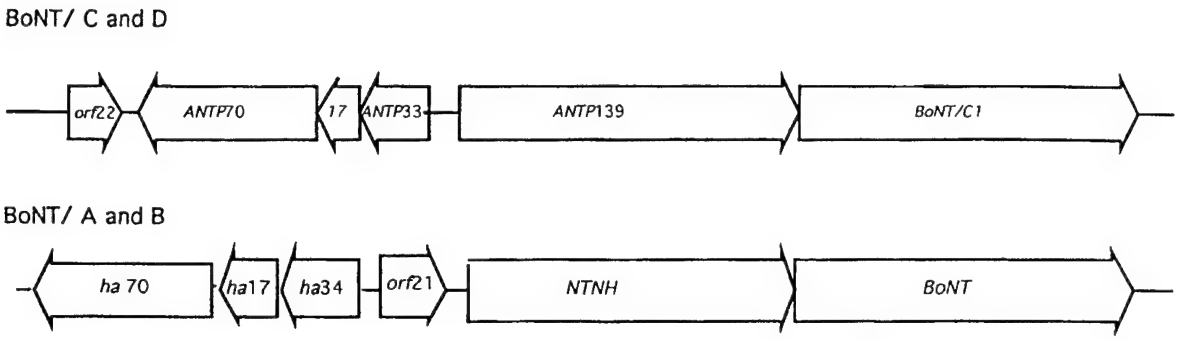


Figure 1. Organization of the botulinum neurotoxin complex genes of *C. botulinum* **A, B, C and D.**

encompasses two genes of approximately the same length: the BoNT/C1 gene localized at the 5' end of the locus, and ANTP139 gene. The close linkage between these two genes and the absence of any terminator-like structure between them suggests that they form an operon. Northern blot analysis showed two mRNA species (approximately 4 and 8 kb), one corresponding to the BoNT/C1 and the other to ANTP/139 and BoNT/C1 genes (unpublished). By reverse transcriptase-PCR, it was confirmed that a mRNA overlapped ANTP/139 and BoNT/C1 genes (Hauser *et al.*, 1994). The transcription start sites of antp139 and bont/C1 genes were determined by primer extension. No consensus promoter sequences could be found upstream from the identified transcription start sites. Therefore, the BoNT/C1 gene can be transcribed alone or in association with antp139 gene.

The three genes (ANTP33, ANTP17 and ANTP70) of the cluster 2 are transcribed in the opposite direction to that of the cluster 1 genes. A mRNA overlapping these three genes has been demonstrated using reverse transcriptase-PCR but these three genes can also be transcribed independently, since a putative terminator sequence is localized at the end of each gene (Hauser *et al.*, 1994).

The final gene (*orf22*) lies upstream of cluster 2 and is transcribed in the same orientation as that for cluster 1.

BoNT locus in other toxinotypes and the deduced proteins

The same genetic organization was found in *C. botulinum* D (Ohyama *et al.*, 1995). The BoNT/A and BoNT/B complex genes are organized differently, the orf21 gene being localized upstream from the antp139 gene (East *et al.*, 1994) (Fig. 1). The full determination of the BoNT complex locus from the other toxin types has not yet been done.

The *antp*139 gene is conserved in all the *C. botulinum* types (reviewed in Popoff and Eklund, 1995). The deduced proteins (139 kDa) are of an equivalent size to the BoNTs, however they do not possess either the Cys residues able to form an interchain disulfide bridge or the metalloprotease motif (HExxH). The ANTP139 (or NTNH) are better conserved (55 to 99% identity at the amino acid level) than the BoNTs (34 to 96%). They could play an important role in the structure of the BoNT complex, and may represent the main component responsible for resistance to low pH and protease degradation.

The ANTP33 (or HA33) from *C. botulinum* C possesses an hemagglutinin activity (Tsuzuki, 1990). The corresponding proteins from *C. botulinum* A and B are weakly related to ANTP33 (36 to 40% identity) (East *et al.*, 1994). ANTP70 and ANTP17 could be additional hemagglutinin components. ANTP70 seems to be cleaved in 23 and 48 kDa proteins. The N-terminal part of the 48 kDa protein shows a significant homology with the *Clostridium perfringens*, enterotoxin which is a pore forming toxin (Hauser *et al.*, 1994).

Orf21 and Orf22 are basic proteins (pI 10.4) typical of DNA binding proteins, and exhibit a 29% identity with the *C. perfringens* regulatory protein Uvia (Hauser *et al.*, 1994), and a 25% identity with a *C. difficile* putative regulatory protein, its gene being located upstream of the toxin A and B genes (Johnson, personal communication).

Genetic approaches in *C. botulinum*

Genetic approaches (construction of defined mutants, gene inhibition ...) are an ideal way to investigate the function of the ANTPs. However, genetic methodologies are poorly developed in *C. botulinum*. Only two reports have been published on gene transfer in *C. botulinum* concerning the conjugal transfer of the transposon Tn916 and plasmid transformation by electroporation (Lin *et al.*, 1991; Zhou *et al.*, 1993). We studied the transformation of *C. botulinum* by electroporation.

The competent cells from *C. botulinum* A NCTC 2916 were prepared in an anaerobic chamber. Bacteria from a mid-log-phase TGY culture (100 ml) were centrifuged, washed in distilled water, and suspended in 0.5 ml of 7 mM phosphate buffer, pH 7.4, containing 1 mM $MgCl_2$ and 270 mM sucrose. Plasmid DNA (1 to 5 mg) was added to 50 ml of cell suspension. Electroporation was performed using a Bio-Rad gene pulser (10 $kV.cm^{-1}$, 200 W and 25 mF) and a cuvette hermetically closed and containing an anaerobic atmosphere. Then, the bacteria were diluted in TGY and plated onto TGY agar containing 5 mg/ml erythromycin in an anaerobic chamber.

Plasmids pGK12 and pAT18 which are transferable to a large variety of Grampositive bacteria (Trieu-Cuot *et al.*, 1991; Zhou and Johnson, 1993) were used. The transformation efficiency of *C. botulinum* NCTC 2916 with pGK12 and pAT18 isolated from *E. coli* HB101 was 102-103 and 10-102 transformants per mg of DNA, respectively. Similar results were reported with pGK12 and a different *C. botulinum* strain (Zhou and Johnson, 1993). When pGK12 was extracted from *C. botulinum*, a higher transformation efficiency was obtained (103-104 transformants per mg of DNA). This indicates the presence of a restriction system in *C. botulinum* NCTC 2916. These results show that electroporation is a reliable transformation method to introduce different classes of plasmids into *C. botulinum*.

Evidence that Orf21 is a positive regulator of BoNT/A gene expression

Genetic analysis suggests that orf21 in *C. botulinum* C and D, and *orf*22 in *C. botulinum* A and B are regulatory genes involved in the control of the expression of the botulinum complex genes. To investigate the function of Orf21 we first overexpressed its gene in *C. botulinum* A. A DNA fragment encompassing *orf*21 and its promoter region was amplified by PCR from *C. botulinum* NCTC 2916, and was cloned into pAT18. The resulting plasmid (pMRP279) was transferred into *C. botulinum* strain NCTC 2916. The copy number of pAT18 was found to be from 100 to 200 per bacteria. The BoNT production was monitored by mouse lethal activity assay in the culture supernatant fluids of the wild type strain and of the strain harboring pMRP279. As shown in Fig. 2, the mouse lethal activity was higher in the recombinant strain than in the wild type. The difference was greater in the exponential phase (102) than in the stationary phase (3–fold) of the culture. These results show that overexpression of *orf*21 increases the BoNT production and suggests that Orf21 is a positive regulator. Since the BoNT/A complex genes are located on *C. botulinum* chromosome and *orf*21 is located on a small plasmid in the recombinant strain, Orf21 acts either in cis or in trans. The small difference in the BoNT production between the wild type and recombinant strains at the stationary phase could be explained by the fact that, in the wild type strain, the regulation system is near the equilibrium. Orf21 could also less effective when its gene is localized in *trans* on a small plasmid.

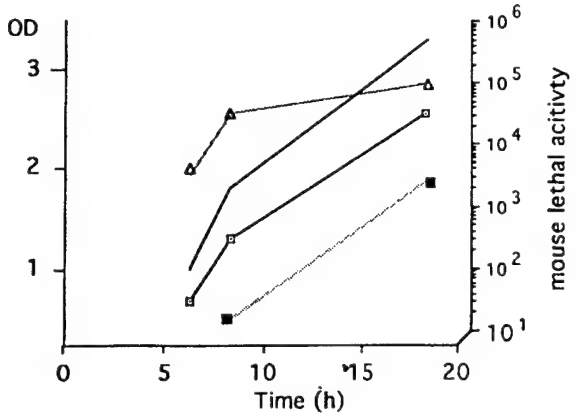


Figure 2. Mouse lethal activity of *C. botulinum* wild type strain (□), strain overexpressing *orf*21 (▲), and strain producing antisense mRNA of *orf*21 (■). The unbroken line indicates the OD.

Analysis of mRNA by dot blot hybridization showed a 2–to 4–fold increase of specific mRNA of *bont*/A and *antp*139 in the recombinant strain. This suggests that *orf*21 regulates not only *bont*/A but also *antp*139 genes.

To confirm the role of *orf*21 we usd an antisense mRNA method to inhibit its expression. A 73 nucleotide fragment encompassing the ribosome binding site and the 18 5' codons was amplified by PCR and was cloned in the reverse orientation into pAT18, downstream the *C. perfringens* iota toxin gene (Perelle *et al.*, 1993). The recombinant plasmid pMRP306 was introduced in *C. botulinum* NCTC 2916 by electroporation. The mouse lethal activity was significantly decreased in the recombinant strain compared to the wild type (Fig. 2).

Taken together, these results indicate that Orf21 is a positive regulator of *bont*/A and presumably of the *antp* genes. The *orf*21 expression blockage by antisense mRNA reduced the expression of *bont*A gene. It is not yet known whether Orf21 acts directey on the promoter region of *bont* and *antp* genes or whether it is involved in a regulatory cascade.

REFERENCES

East AK, Stacey J M and Collins M (1994) Cloning and sequencing of a hemagglutinin component of the botulinum neurotoxin

complex encoded by *Clostridium botulinum* types A and B. *System. Appl. Microbiol.* **17**, 306-312.

Hauser D, Eklund MW, Boquet P and Popoff MR (1994) Organization of the botulinum neurotoxin C1 gene and its associated non-toxic protein genes in *Clostridium botulinum* C468. *Mol Gen Genet* **243**, 631-640.

Lin W and Johnson EA (1991) Transposon Tn916 mutagenesis in *Clostridium botulinum*. *Appl. Environ. Microbiol.* **57**, 2946-2950.

Ohyama T, Watanabe T, Fujinaga Y, Inoue K, Sunagawa H, Fujii N, Inoue K and Oguma K (1995) Characterization of nontoxic-nonhemagglutinin component of the two types of progenitor toxin (M and L) produced by *Clostridium botulinum* type C CB-16. *Microbiol Immuno.* **39**, 457-465.

Perelle S, Gibert M, Boquet P and Popoff MR (1993) Characterization of *Clostridium perfringens* iota toxin genes and expression in Escherichia coli. *Infect Immun* **61**, 5147-5156.

Popoff MR and Eklund MW (1995) Tetanus and botulinum neurotoxins: genetics and molecular mode of action. In Eklund MW, Richards J and Mise K (eds): *Molecular approaches to food safety, issues involving toxic microorganisms.* Fort Colins, Alaken: pp. 481-511.

Trieu-Cuot P, Carlier C, Poyart-Salmeron C and Courvalin P (1991) Shuttle vectors containing a multiple cloning site and a *lacZa* gene for conjugal transfer of DNA from *Escherichia coli* to Gram-positive bacteria. *Gene* **102**, 99-104.

Tsuzuki K, Kimura K, Fujii N, Yokosawa N, Murakami IT, and Oguma, K (1990) Cloning and complete nucleotide sequence of the gene for the main component of hemagglutinin produced by *Clostridium botulinum* type C. *Infect Immun* **58**, 3173-3177.

Zhou Y and Johnson EA (1993) Genetic transformation of *Clostridium botulinum* Hall A by electroporation. *Biotech. Letts* **15**, 121-126.

Structure and function of progenitor toxins produced by *Clostridium botulinum* types A, C, D, and E

Keiji Oguma[1*], Yukako Fujinaga[1], Kaoru Inoue[1], Ken-ichi, Tomochika[1], Toshihiro Watanabe[2], Kouichi Takeshi[3] Tohru Ohyama[3] and Katsuhiro Inoue[2]

[1]Department of Bacteriology, Okayama University, Medical School, 2-5-1 Shikata-cho, Okayama 700, JAPAN, [2]Department of Food Science, Faculty of Bioindustry, Tokyo University of Agriculture, 196 Yasaka, Abashiri 099-24, JAPAN, and [3]Hokkaido Institute of Public Health, N.19., W.12., Sapporo 060, Japan

Keywords: C. botulinum, progenitor toxin, toxin structure, haemagglutinin, nontoxic component, gene cloning, gene organisation, absorption, binding

The molecular compositions of the progenitor toxins, 12S, 16S and 19S have been analysed and the following conclusions were drawn: 1) The 12S toxin consists of a neurotoxin and a nontoxic component having no haemagglutinin (HA) activity designated as nontoxic-nonHA; 2) The 16S and 19S toxins are formed by conjugation of the 12S toxin with HA; 3) the nontoxic-nonHAs of 19S and 16S toxins are single peptides but those of 12S toxins of types A, C and D have a cleavage on their N-terminal regions within a short repeat sequence motif. Type E (and F) nontoxic-non HA has a deletion of this second repeat sequence motif; 4) HA consists of subcomponents having Mr of approximately 53, 33, 21–22 and 17 kDa. HA-33 and -17 are the products of their respective genes but HA-53 and HA-21–22 are formed by the processing of a *ha-70* gene product (70 k); 5) the molar ratio of neurotoxin, nontoxic-nonHA and HA (53, 33, 21~22 and 17) of the 16S toxin is approximately 1 : 1 : 2 x (1 : 2: 1: 1). Type A 19 S toxin may consist of two molecules of the 16 S toxin which are cross-linked by HA-33; and 6) type C 16 S toxin can bind to the epithelial cells of guinea pig-small intestine but the 7S and 12S toxins hardly bind to them.

Introduction

Clostridium botulinum strains produce immunologically distinct neurotoxins (type A to G). The molecular masses of all types of neurotoxins are approximately 150 kDa. The neurotoxins associate with nontoxic components in the culture fluids and form large complexes which are designated as progenitor toxins. In type A, three different-sized progenitor toxins with molecular masses of 900 kDa (19S) 500 kDa (16S) and 300 kDa (12S were produced). The type B, C and D strains produced the 16S and 12S toxins, whereas types E and F and type G produce only the 12S or 16S toxins, respectively (Sakaguchi *et al.*, 1984). In all of these types, the nontoxic components of the 19S and 16S toxins have haemagglutinin (HA) activity but that of the 12S toxin does not. Therefore, it was postulated that the 12S toxin is formed by association of a neurotoxin with a nontoxic component having no HA activity which is designated here as a nontoxic, non-HA and that the 19S and 16S toxins are formed by conjugation of the 12S toxin with HA and that the 19S and 16S toxins are formed by conjugation of the 12S toxin with HA. The nontoxic components are considered to be very important to the development of food poisoning because they protect the neurotoxin from the acidity and proteases in the stomach (Sakaguchi *et al.*, 1984).

The molecular masses of the nontoxic, non-HAs of all types of progenitor toxins have been determined to be approximately 140 kDa by sodium dodecyl sulphate-polyacrylamidine gel electrophoresis (SDS-PAGE). The molecular masses of HAs are not clear. (i) Why do the 12S, 16S and 19S (progenitor) toxins exist in the same culture? (ii) How are the 12S, 16S and 19S toxins formed? (iii) What are the function of

*To whom correspondence should be sent. Keiji Oguma, Department of Bacteriology, Okayama University, Medical School, 2-5-1 Shikata-cho, Okayama 700, JAPAN.
Tel & Fax +81-86-222-1815.

the nontoxic, non-HAs and HAs? In order to answer these questions, the progenitor toxins of types A, C, D and E were purified, and their molecular compositions analysed. In addition, the binding of the type C 7S (neurotoxin), 12S and 16S toxins to the epithelial cells of small intestine and their subsequent absorption, were analysed in guinea pigs.

Materials and methods

Bacterial strains. Laboratory strains employed were; type A, A-NIH, type C, C-Stockholm, type D, D-CB16 and type E E-Iwanai.

Purification of toxins. Bacterial strains were cultivated using the cellophane tube procedure and the toxins were precipitated by 50–60% saturation of ammonium sulphate. Since the type A culture fluid contains RNA, it was removed by protamine treatment (Sugii and Sakaguchi, 1975). The progenitor toxins were purified by successive column chromatography on SP-Toyopearl 650 M (Tosoh, Tokyo, Japan) and Sephacryl S-300 (Pharmacia, Uppsala, Sweden) for type A, or Toyopearl HW-55 for types C and D. All the chromatography was performed at pH 4.0 or 4.2 at room temperature. Separation of type A 19S and 16S toxins were performed by sucrose density gradient centrifugation at pH 6.0.

Determination of toxin and HA titres. The preparations were diluted in serial 10- and/or 3- fold steps with 20 mM sodium phosphate buffer (pH 6.0) containing 0.2% (w/v) gelatin and 0.5 ml of each dilution was intraperitoneally injected into three white mice (about 20 g). The mice were observed for one week and the minimal lethal dose (MLD) per ml was obtained.

The haemagglutinin titre was obtained by micro titration in multi-well plates. The toxin solution (50 l) was diluted in serial 2-fold steps with 10 mM phosphate buffered saline (pH 7.4) and mixed with an equal volume of 2% suspension of washed human erythrocytes (group O). After incubation at room temperature for 2 h, the reciprocal of the highest dilution at which hemagglutination was positive was denoted as the haemagglutinin titre (2n).

SDS-PAGE. SDS-PAGE was performed by the method of Laemmli. In an attempt to analyse the composition of type A progenitor toxins, 5-20% acrylamide gradient gel purchased from Tefco (Tefco Co., Ltd., Nagano, Japan) was employed. On the other hand, 7.5 or 12.5% acrylamide linear gel was used for analysis of the N-terminal amino acid sequences of each component of the progenitor toxins. Protein bands were stained with CBB R-250 (Inoue *et al.*, 1996).

Determination of Amino Acid Sequence. The N-terminal amino acid sequences of each protein were determined. The bands, separated with the 7.5 or 12.5% acrylamide linear gel of SDS-PAGE, were electro blotted to polyvinylidene difluoride (PVDF) membranes ProBlott TM, Applied Biosystems, Foster City, CA) with a semidry blotting apparatus (Nippon Eido, Tokyo, Japan). The bands were stained, cut out and then sequenced by a pulsed-liquid phase protein sequencer (model 477-A, Applied Biosystems, Foster City, CA). All samples were sequenced more than twice (Inoue *et al.*, 1996) .

Densitometrical analysis. Densitometry was performed with a Scanning Imager 300SX (Molecular Dynamics, Sunnyvale, CA). The intensity of the protein band was estimated by the area of the peak. The molar ratio of each band was calculated by dividing the intensity or band by each Mr. The neurotoxin was expressed as 1.0. Measurement was performed three times with different preparations and the means of these values were obtained.

Cloning of toxin genes. In types C and D toxin production is governed by specific bacteriophages. Therefore, the toxin and the nontoxic component genes were cloned from the phage DNAs (Kimura *et al.*, 1990). Phage DNA libraries were constructed in gt 11 by using *Eco*RI and were transformed into *E. coli* Y1090. Colonies were immunoscreened for reactivity with monoclonal antibodies against the neurotoxins as well as a polyclonal antibodies against the non toxic component prepared previously (Oguma *et al.*, 1980; Oguma *et al.*, 1984).

In type C, a 3 kilobase (kb) *Eco*RI fragment, designated as CH3, which contained the 3' end of the neurotoxin gene (*ctx*), and a 7.5 kb *Eco*RI fragment, designated as CL8, which contained the 5' end of the *ctx*, as well as the entire sequences of the non toxic non-HA (*cnt*), HA-33 (*cha-33*) and HA-17 (*cha-17*) genes were obtained. In addition, a 2.5 kb *Hind*III fragment, designated as CHA, which hybridised with the 300 bp *Eco*RI- *Hind*III fragment from the CL8, was obtained (Fujinaga *et al.*, 1994). CHA contained the *cha-70* gene, encoding both HA 22-23 and HA-53. In type D, a 9.8 kb *Eco*RI fragment, designated D-3, which contained ORFs for neurotoxin, non toxic non-HA, HA-33, HA-17 and HA 22-23 was isolated (Sunagawa *et al.*, 1992; *Ohyama et al.*, 1995). The genes comprising type E *C. botulinum* toxin were also immunoscreened by antisera specific for the neurotoxin and non-toxic non-HA from chromosomal DNA libraries constructed in pUC119. A 6kb *Hind*III fragment encoding the entire non toxic non-HA, entire L chain and the N-terminal region of H-chain of the neurotoxin was obtained (Fujii *et al.*,

1993). In type A, a 4.6 kb fragment containing the non toxic non-HA gene and surrounding region was amplified by PCR with the primers pAHR3 and pAR3, corresponding the coding regions for N-terminals of the HA-35 gene and the neurotoxin gene (Fujita *et al.*, 1995). The PCR products were cloned directly into pT7 Blue T-Vector (Novagen, Madison WI, USA).

Determination of nucleotide sequences. DNA was sequenced on both strands by the dideoxychain-terminating method. For type A, the sequence was determined on two cloned fragments derived from different PCR experiments. Where the two clones differed in sequence, a third independently amplified fragment was cloned and sequenced.

Toxin binding to epithelial cells

1) in vivo test

Guinea pigs (8-12 weeks of age, fasted for 24h) were anaesthetized and the intestine was exposed by a midline incision. Ligated intestinal loops (3-4cm in length) were prepared at the upper small intestine, lower small intestine, proximal colon, and distal colon. Each loop was inoculated with 0.2-0.3ml of toxin solutions by using a 27-gauge needle. Loops were incubated for 1h in the abdominal cavity. The loops were excised from the guinea pigs, opened and briefly washed in two changes of ice-cold PBS (pH 6) and fixed in periodate-lysine-paraformaldehyde (PLP) fixative. The tissues were dehydrated and paraffin-embedded before cutting 5μ thick sections. The sections were dewaxed and stained with rabbit anti-type C 16S toxin followed by biotinylated goat anti-rabbit (Vector Laboratories, Burligame, Ca., USA). The slides were developed using avidin-biotin peroxidase complexes (Vector Laboratories) and DAB substrate.

2) in vitro test

Samples of the upper and lower small intestine and the proximal and distal colon were removed from the anaesthetized guinea pigs. All samples were fixed with 4% paraformaldehyde fixative, dehydrated and embedded in paraffin. Sections 5μ thick) were dewaxed and treated with the toxin in a humidity chamber for 1h at room temperature. They were then washed in six changes of PBS (pH 6)- o.1% Triton X-100 for 10 min and stained with rabbit anti-type C 16S toxin as described above.

Absorption of progenitor toxin in small intestine

Guinea pigs were anaesthetized and the intestine exposed via a smalll midline excision. A 0.4ml dose of toxin was injected into the upper small intestine with a 1ml syringe attached to a 27 gauge needle. After the incision was sutured, the guinea pigs were kept in restraining cages for 6-8h. Blood was collected by cardiocentesis and the resulting sera serially diluted. Each diluted preparation (0.5ml) was injected *ip* into 3 mice and the MLD per ml calculated.

RESULTS AND DISCUSSIONS

Composition of progenitor toxins

The 12S, 16S and 19S toxins were purified from A-NIH and the 12S and 16S toxins were purified from C-Stockholm and D-CB16 whereas only the 12S toxin was obtained from E-Iwanai. On SDS-PAGE analysis with or without 2-ME type E 12S toxin demonstrated 144 and 133 kDa bands which correspond to the neurotoxin and the nontoxic, non-HA respectively (Yokosawa *et al.*, 1986). Type C 12S toxin demonstrated three bands of 150, 115 and 18 kDa in the absence of 2-ME, whereas four bands of 100, 50, 115 and 18 kDa were evident in the presence of 2 ME. Based on the N-terminal amino acid sequences determined by protein chemistry and genetical analysis, it was concluded that 150 kDa protein was the neurotoxin and 100 and 50 kDa proteins are its heavy (H) and light (L) chain components. The 115 and 18 kDa proteins are derived from the nontoxic, non-HA (140 kDa) by cleavage within its N-terminal region. In the case of type D 12S toxin, the nontoxic, non-HA separated into 115 and

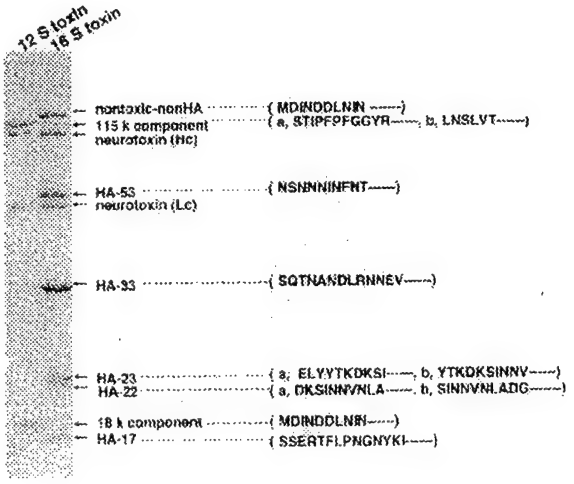


Figure 1 SDS-PAGE patterns and N-terminal amino acid sequences of each subcomponent of type C 12S and 16S toxins. The purified type C 12 S and 16 S toxins were heated at 100°C for 7 min in the presence of 2-ME, and electrophoresis was performed on a 12.5% polyacrylamide linear gel. N-terminal amino acid sequences of each band are shown by the single-letter code.

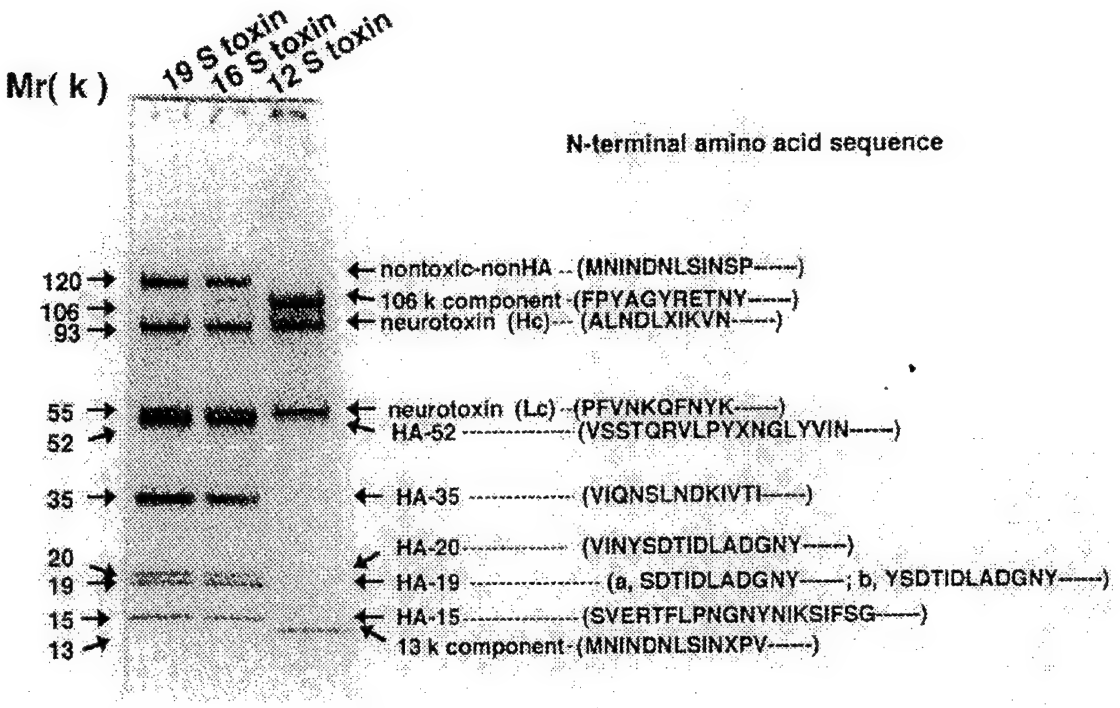


Figure 2 SDS PAGE patterns and N-terminal amino acid sequences of type A progenitor toxins. The purified type A 12 S, 16 S and 19 S toxins were heated at 100° C for 7 min in the presence of 2-ME, and electrophoresis was performed on a 5 to 20 % polyacrylamide gradient gel. N-terminal amino acid sequences of each band are shown by the single-letter code.

15 kDa proteins. Both type C and D 16S toxins demonstrated 53, 33, 22-23 and 17 kDa bands besides the neurotoxin and nontoxic, non-HA bands indicating that the HA consists of four subcomponents. This was confirmed by the amino acid sequences. The open reading frames (ORFs) coding for HA-33 and HA-17 were identified, while those for HA-53 and HA-22-23 were not (Fujinaga *et al.*, 1994). Downstream of *cha-17* the gene (*cha-70)* encoding a 623 amino residues was identified. It was concluded that the produce of *cha-70* is split into HA-53 and HA22-23 after translation and that the N-terminal region of HA-22-23 is cleaved within at least four different sites to form proteins with slightly different Mrs (Fig. 1). Similar results were obtained in type A (Fig. 2). The nontoxic, non-HA of the 12S toxin was cleaved within its N-terminal region to form two fragments (106 and 13 kDa). The 16S and 19S toxins demonstrated the same banding profiles, indicating that they consist of the same protein components. The HA consists of four subcomponents with molecular weights of 52, 35, 19–20 and 15 kDa. The HA-19–20 consists of several proteins with slightly different Mrs. Molar ratios of the neurotoxin, nontoxic, non-HA and each HA subcomponent of 53, 19–20 and 15 kDa are 1 : 1 : 2 (Table 1). Molar ratios of the neurotoxin, HA-35 of the 16S toxin and HA-35 of the 19S toxin are 1 : 4 : 8. Therefore, we conclude that the 19S toxin is a dimer of the 16S toxin cross-linked by 4 molecules of HA-35 (Fig. 3).

Table 1. Densitometry of each component of the 16S and 19S progenitor toxins.

Protein	*Molar Ratio*	
	19S	16S
neurotoxin	1.0	1.0
HA-52	2.2 (2)	2.6 (2)
HA-35	7.8 (8)	3.9 (4)
HA-19~20	3.4 (2)	3.3 (2)
HA-15	2.7 (2)	2.6 (2)
nontoxic non-HA	1.4 (1)	1.2 (1)

The molar ratios were calculated by dividing the intensity of each protein band by each molecular mass. The molar ratio of neurotoxin was expressed as 1.0. Electrophoresis was performed in the presence of 2-ME. Since HA-19 and HA-20 could not be separated by the densitometer, the ratio of these subcomponents were calculated as one component. The values are the mean of three recordings. The estimated molar ratios are described in parentheses.

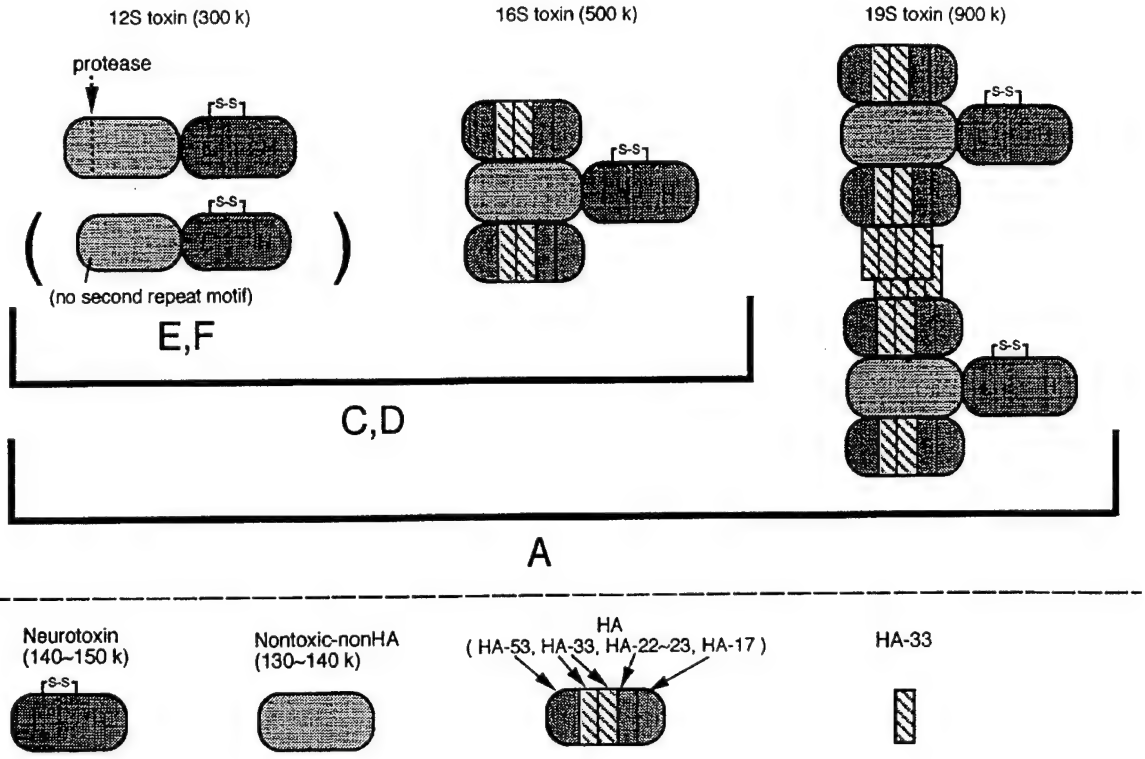


Figure 3. Scheme of the progenitor toxins

Amino acid alignment of the N-terminal regions of nontoxic non-HAs of types A, C, D, E and F

Percentage amino acid identities of the nontoxic, non-HAs among *C. botulinum* types A to F are 55 (between C or D and E) to 99 (between C and D). A multiple alignment of N-terminal regions is shown in Fig. 4. As described above, the nontoxic, non-HAs of 12S toxins of types A, C and D have a cleavage after the Pro 144, Thr 140 and Thr 140 respectively. These residues are contained in the region that includes a short repeat of the sequence and the cleavages occur in the second repeat motif. On the other hand, type E and F nontoxic, non-HAs lack the second repeat

```
Type A*     61:GGIYDSNFLSQDSEKDKFLQAIITLLKRINSTNAGEKLLSLISTAIPFPYGYIGGGYYAP 120
Type C*     61:GGIYDSNFLSQDSERENFLQAIIILLKRINNTISGKQLLSLISTAIPFPYGYIGGGYSSP 120
Type D*     61:GGIYDSNFLSQDSERENFLQAIIILLKRINNTISGKQLLSLISTAIPFPYGYIGGGYSSP 120
Type E*     60:GGIYDSNFLSTNNEKDDFLQATIKLLQRINNNVVGAKLLSLISTAIPFPYFNNT------ 113
Type F**    61:GGIYDENFLKENSEKEEFLQAIILLLKRINNNIIGQKLLSLMCTSIPFLHEYKQ------ 114

Type A     121:NMITFGSAPKSNKKLNSLISSTIPFPYAGYRETNYLSSEDNKSFYASNIVIFGPGANIVE 180
Type C     121:NIFTFGKTPKSNKKLNSLVTSTIPFPFGGYRETNYIESQNNKNFYASNIIIFGPGSNIVE 180
Type D     121:NIFTFGKTPKSNKKLNSLVTSTIPFPFGGYRETNYIESQNNKNFYASNIVIFGPGSNIVE 180
Type E     114:----------------------------EDYRQTNYLSSKNNEHYYTANLVIFGPGSNIIK 146
Type F     115:----------------------------GDYRQSNYLGSKNSEYLYSANIVIFGPGSNIVK 147
```

*, our data ; ** East et al., (1994) ; ▼, cleavage site (A, Pro[144]-Phe; C, Thr[140]-Ser; D, Thr[140]-Ser)

Figure 4 Amino acid alignment of the N-terminal regions of nontoxic-nonHAs of types A, C, D, E and F. The repeated motifs are underlined. The arrowheads indicate N-terminal amino acids of ~100 kDa components of 12 S toxins determined experimentally by protein sequencing.

motif. These results indicate that the region in and around the second repeat motif may have a critical role in forming HA-positive progenitor toxins by conjugation of the nontoxic non-HA components with the HAs.

Binding and absorption of the toxins in guinea-pig intestine

Binding of the purified type C neurotoxin, 12 S and 16S toxins to the epithelial cells of ligated small intestines or colons of guinea pigs (*in vivo* test), and to sliced tissue preparations (*in vitro* test) was analysed. The 16 S toxin strongly bound to the microvilli of the epithelial cell of small intestines in both *in vivo* and *in vitro* tests, but hardly bound to the cells of colons. On the other hand, the neurotoxin and the 12 S toxin hardly bound to the epithelial cells of either small intestines or colon. Absorption of the toxins was checked by determining the toxin titre in the sera of guinea pigs 6 to 8 h after the intra-intestinal administration of the toxins. When the 16 S toxin (1×10^5 MLD/0.4 ml) was injected, 200 MLD/ml was detected in the sera, whereas when the 12 S (2×10^5 MLD/0.4 ml) was injected, 0.5 ml of the three times diluted sera did not kill mice. Therefore, we concluded that the HA component of type C 16 S toxin is very important in the binding (and absorption) step in the small intestine.

Acknowledgment

We thank Kiyomi Hamada and Rui Fujita for technical assistance.

References

East AK, Richardson PT, Allaway D, Collins MD, Roberts TA and Thompson DE (1992) Sequence of the gene encoding type F neurotoxin of *Clostridium botulinum*. *FEMS Microbiol. Lett.* 75, 225-230.

Fujii N, Kimura K, Yokosawa N, Yashiki T, Tsuzuki K and Oguma K (1993) The complete nucleotide sequence of the gene encoding the nontoxic component of *Clostridium botulinum* type E progenitor toxin. *J. Gen. Microbiol.* **139**, 79-86.

Fujinaga Y, Inoue K, Shimazaki S, Tomochika K, Tsuzuki K, Fujii N, Watanabe T, Ohyama T, Ohyama K, Takeshi K, Inoue K and Oguma K (1994) Molecular construction of *Clostridium botulinum* type C progenitor toxin and its gene organization. *Biochem. Biophys. Res. Commun.* **205**, 1291-1298.

Fujita R, Fujinaga Y, Inoue K, Nakjima H, Kumon H and Oguma K (1995) Molecular characterization of two forms of nontoxic-nonhemaggulutinin components of *Clostridium botulinum* type A progenitor toxins. *FEBS Lett.* **376**, 41-44.

Inoue K, Fujinaga Y, Watanabe T, Ohyama T, Takeshi K, Moriishi K, Nakajima H, Inoue K and Oguma K (1996) Molecular composition of *Clostridium botulinum* type A progenitor toxins. *Infect. Immun.* **64**, 1589-1594.

Kimura K, Fujii N, Tsuzuki K, Murakami T, Indoh T, Yokosawa N, Takeshi K, Syuto B, and Oguma K (1990) The complete nucleotide sequence of the gene coding for botulinum type C1 toxin in the C-ST phage genome. Biochem. Biophys. Res. Commun. **171**, 1304-1311.

Oguma K, Syuto B, Iida H, and Kubo S (1980) Antigenic similarity of toxins produced by *Clostridium botulinum* type C and D strains. *Infect. Immun.* **30**, 656-660.

Oguma K, Murayama S, Syuto B, Iida H, and Kubo S (1984) Analysis of antigenicity of *Clostridium botulinum* type C1 and D toxins by polyclonal and monoclonal antibodies. *Infect. Immun.* **43**, 584-588.

Ohyama T, Watanabe T, Fujinaga Y, Inoue K, Sunagawa H, Fujii N, Inoue K, and Oguma K (1995) Characterization of nontoxic-nonhemaggulutinin component of the two types of progenitor toxin (M and L) produced by *Clostridium botulinum* type D CB-16. *Microbiol. Immunol.* **39**, 457-465.

Sakaguchi G and Ohnishi I (984) Structure and function of botulinum toxins. In J. E. Alouf, F. J. Fehrenbach, J. H. Freer and J. Jeljaszewicz (eds) *Bacterial Protein Toxins.* Academic Press, London; pp435-443.

Sugii S and Sakaguchi G (1975) Molecular construction of *Clostridium botulinum* type A toxins. *Infect. Immun.* **12**, 1262-1270.

Sunagawa H, Ohyama T, Watanabe T and Inoue K (1992) The complete amino acid sequence of the *Clostridium botulinum* type D neurotoxin, deduced by nucleotide sequence analysis of the encoding phage d-16 pfi genome. *J. Vet. Med. Sci.* **54**, 905-913.

Tsuzuki K, Kimura K, Fujii N, Yokosawa N, Indoh T, Murakami T and Oguma K (1990) Cloning and complete nucleotide sequence of the gene for the main component of hemagglutinin produced by *Clostridium botulinum* type C. *Infect. Immun.* **58**, 3173-3177.

Tsuzuki K, Kimura K, Fujii N, Yokosawa N and Oguma K (1992) The complete nucleotide sequence of the gene coding for the nontoxic-nonhemagglutinin component of *Clostridium botulinum* type C progenitor toxin. *Biochem. Biophys. Res. Commun.* **183**, 1273-1279.

Yokosawa N, Tsuzuki K, Syuto B and Oguma K (1986) Activation of *Clostridium botulinum* type E purified by two different procedures. *J. Gen. Microbiol.* **132**, 1981-1988.

Phylogenetics of *Clostridium botulinum* and the botulinum neurotoxins

M. D. Collins and A. K. East

Department of Microbiology, Institute of Food Research, Reading, RG6 6BZ, United Kingdom

Keywords: *Clostridium botulinum*, botulinum neurotoxin, nontoxic-nonhemagglutinin, phylogenetics.

16S rRNA sequencing studies have revealed the presence of four phylogenetically distinct lineages within *Clostridium botulinum*, which correlate with the four recognized divisions (groups I-IV) within this so-called species. In addition to marked phenotypic and genotypic heterogeneity, the taxonomy of the species is further complicated by the existence of strains which are genetically identifiable as *C. botulinum* but are non-toxigenic, and, strains of species (viz., *C. baratii, C. botulinum*) other than *C. botulinum* which produce botulinum neurotoxin (BoNT). Genealogical trees derived from BoNTs show marked discordance with those depicting 'natural' relationships inferred from 16S rRNA. Strong evidence exists for BoNT gene transfer between some groups of *C. botulinum* (e.g., groups I and II), and with non-botulinum species. Information on the phylogenetics of the genes encoding nontoxic-nonhaemagglutinin (NTNH), located immediately upstream of that of BoNT, is currently limited. However, there is clear evidence of chimera-like or mosaic NTNH genes in group I *C. botulinum* It is apparent that the phylogeny of the NTNHs is not going to 'mirror' that of botulinum neurotoxins, although their genes are physically contiguous.

Taxonomy and phenotypic divisions of *Clostridium botulinum*

C. botulinum is the taxonomic designation used for all organisms known to produce botulinum neurotoxin (BoNT) and thereby having the capability of causing botulism in humans and animals (Prevot, 1953). Strains of *C. botulinum* are traditionally classified into seven types (A through G), depending on the serological specificity of the neurotoxin produced (Hatheway, 1990, 1992). Defining *C. botulinum* solely on the basis of BoNT production has, however, resulted in the species encompassing a range of metabolically diverse microorganisms (Hatheway, 1990, 1992). Holdeman and Brooks (1970) divided *C. botulinum* types A to F into three metabolic groups. Strains of type G, described by Giménez and Ciccarelli (1970), were subsequently placed in a fourth group by Smith and Hobbs (1974). Thus four physiologically distinct groups (designated I to IV) are recognized within *C. botulinum*. From a purely taxonomic viewpoint, it is now recognized that a nomenclature based on BoNT production is unsatisfactory, and that *C. botulinum* as currently defined consists of several species. The taxonomy of *C. botulinum* has been further complicated in recent years by reports that some strains of *Clostridium butyricum* and *Clostridium baratii* produce neurotoxins that cross-react with BoNT types E (Aureli *et al.*, 1986; McCroskey *et al.*, 1986) and F (Hall *et al.*, 1985; McCroskey *et al.*, 1991) respectively.

Corresponding author: Department of Microbiology, Institute of Food Research, Earley Gate, Whiteknights Road, Reading, RG6 6BZ United Kingdom: Tel: +44 1734 357225; Fax: +44 1734 267917

Phylogenetic divisions within *C. botulinum* based on 16S rRNA

It is only with the advent of 16S rRNA gene sequencing that the full extent of the genealogical heterogeneity within the *C. botulinum* species-complex has become apparent (Hutson *et al.*, 1993a,b). The phylogenetics of the genus *Clostridium* has been systematically investigated in recent years (Collins *et al.*, 1994) and it is now evident that the genus is not a monophyletic group but actually consists of >20 genera and embraces several families. Sequencing of 16S rRNA (Hutson *et al.*, 1993a,b; Collins *et al.*, 1994) has shown *C. botulinum* groups I-IV and other clostridial species producing BoNT are members of a clade designated cluster I which corresponds to the genus *Clostridium sensu stricto*. Within rRNA cluster I organisms of *C. botulinum* phenotypic groups I-IV

form four distinct phylogenetic lineages (Hutson *et al.*, 1993b). All strains regardless of toxin type within proteolytic or group I *C. botulinum* (i.e. types A, B and F) are highly related to each other (*ca.* 99.7 to 100% 16S rRNA sequence similarity), and together with their non-toxigenic counterparts form a single phylogenetic unit. The closest phylogenetic relative of group I *C. botulinum* and *Clostridium sporogenes* is *Clostridium oceanicum*. Similarly, non-proteolytic or group II *C. botulinum* types B, E and F (and non-toxigenic variants) form a distinct line quite separate from other saccharolytic clostridia and are phylogenetically far removed from group I *C. botulinum*. Sequences of 16S rRNA show group III *C. botulinum* types C and D are phylogenetically closely related (*ca.* 99% similarity) and represent a third lineage. Although types C and D organisms are highly related, the reported 1% sequence divergence between the 2 strains examined is strongly indicative of species heterogeneity within group III organisms. *Clostridium novyi* has been shown to be a close phylogenetic relative, albeit a different species, from group III *C. botulinum*. Surprisingly, two thermophilic clostridia, *Clostridium thermopalmarium* and *Clostridium thermobutyricum*, possess a distant but significant affinity with group III *C. botulinum*. Group IV *C. botulinum* is phylogenetically quite separate from the other three *C. botulinum* groups. *Clostridium subterminale* is phylogenetically a close relative of *C. botulinum* type G with some strains being genealogically indistinguishable. With the possible exception of a loose association with *Clostridium estertheticum*, *C. botulinum* type G and *C. subterminale* show no specific affinity with any other clostridial species (Hutson *et al.*, 1993b; Collins *et al.*, 1994). Sequences of 16S rRNA have also shown that the BoNT synthesizing strains of *C. baratii* and *C. butyricum* are genealogically indistinguishable from non-toxigenic strains (including type strains) of their respective species (Hutson *et al.*, 1993a, b). *Clostridium tetani* which produces tetanus toxin (TeNT), although also a member of cluster I *Clostridium*, is phylogenetically quite distinct from the four *C. botulinum* groups including toxigenic *C. baratii* and *C. butyricum*. A tree depicting the phylogenetic interrelationships of the various *C. botulinum* types and some cluster I clostridial species is shown in Fig. 1. It is evident from the tree that the phylogenetic subdivisions of *C. botulinum* based on 16S rRNA directly 'mirror' the sub-divisions (I-IV) based upon phenotype.

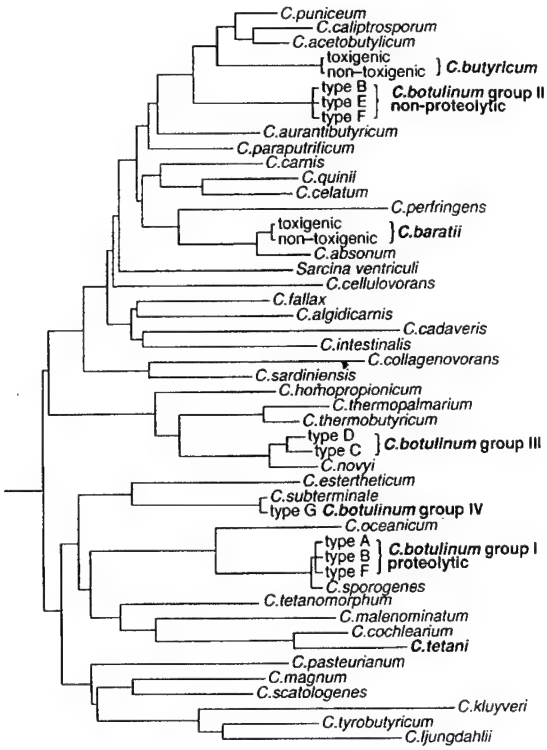


Figure 1. Dendrogram showing the phylogenetic position of *C. botulinum* groups I-IV within cluster I *Clostridium* based on 16S rRNA sequences.

Phylogenetic relationships of BoNTs

The past few years has seen the publication of many BoNT gene sequences, which complete sequences for all *C. botulinum* types (A through G) now elucidated. Comparative analysis has shown that all BoNT types closely resemble each other, and their similarity to tetanus toxin (tetanospasmin, TeNT) is striking (Elmore *et al.*, 1995). Figure 2 shows two trees depicting the phylogenetic interrelationships of the L and H chains of the various BoNT types. It is evident that the BoNT and 16S rRNA derived trees are incompatible, and that major topological differences reinforce the discordance between toxin type and genotypic (natural) divisions with the *C. botulinum* species complex. The most significant finding to emerge from comparative phylogenetic analyses is the strong likelihood of BoNT gene transmissibility within the *C. botulinum* species complex and also with non-botulinum species. In particular, the BoNT/E's of *C. botulinum* and *C. butyricum* are very highly related (*ca.* 97% amino acid identity), as are BoNT/B's of proteolytic (group I) and non-proteolytic (group II) *C. botulinum* (*ca.* 93% amino acid identity). Such exceptionally high levels of sequence relatedness are clearly indicative of recent lateral gene transfer between these genotypes. Similarly, it is evident from 16S rRNA sequence analysis that BoNT/F producing organisms (viz: group I and II *C.*

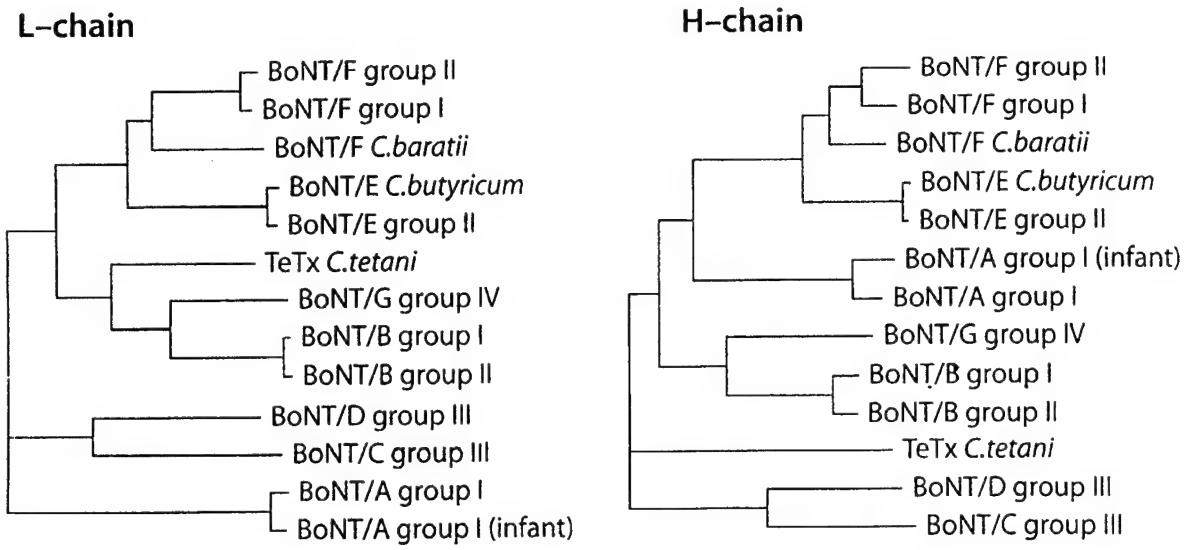


Figure 2. Dendrograms showing the phylogenetic relationships of the L- and H- BoNT chains.

botulinum and *C. baratii*) form 3 quite separate phylogenetic lineages (Fig. 1). However, the type F toxins of these species are of common ancestry, forming a genealogically tight grouping (Fig. 2). The type F toxins from group I and II *C. botulinum* are more closely related to each other (*ca.* 87% amino acid identity) than either are to *C. baratii* (*ca.* 71-74% amino acid identity). It is also apparent from the treeing analysis that the 3 type F toxins are more loosely associated with each other, than are the type E toxins of *C. botulinum* and *C. butyricum*. Although there can be little doubt that the 3 type F toxins are of common ancestry, if BoNT gene transfer has in fact occurred between these 3 species (i.e. *C. botulinum* groups I and II and *C. baratii*), the significantly lower identities indicate such an event occurred earlier in evolutionary history (assuming a similar rate of nucleotide change in the genes). It is evident from comparative sequence analysis that type G toxin, although quite separate from that of type B, nevertheless, exhibits a loose but statistically significant phylogenetic association (in much the same way as BoNT/E and BoNT/F exhibit a distant but significant evolutionary affinity).

Phylogenetic relationships of group I *C. botulinum* NTNHs

Botulinum neurotoxin is produced by *C. botulinum* as a non-covalently bound complex of two or more protein components. The smallest complex, the M-complex (*ca.* 300 KDa) found in all strains consists of BoNT with a similarly sized nontoxic-nonhemagglutinin (NTNH). In addition some strains produce larger complexes (L-complex, *ca.* 500 KDa; LL-complex, *ca.* 900 KDa) which have associated hemagglutinin activity. The genes encoding these non-toxin proteins are clustered with that encoding BoNT. For example, the NTNH gene is located immediately upstream of that encoding BoNT in all toxin types, and genes for other components of complexes have been shown to be clustered upstream of the NTNH gene for toxin types A, B and C. Knowledge of the structural organisation of the cluster of genes encoding BoNT-complex components is currently far from complete. The evolutionary histories of the various progenitor toxin components have also not been systematically investigated but because of their genes proximity to that encoding BoNT, it is not unreasonable to expect their genealogy would mirror that of the BoNTs. Despite a paucity of comparative sequence information, this appears not to be the case for the NTNHs.

Of the various *C. botulinum* organisms, the NTNHs of group I *C. botulinum* have been the most studied, with full gene sequences known for types A, A (B), B, and F. It is evident that for group I *C. botulinum*, the evolutionary patterns of the BoNT and NTNH genes are incompatible. In particular there is evidence of 'chimera-like' NTNH gene sequences. Hutson *et al.* (1996) recently characterized two BoNT gene clusters encoded by type A *C. botulinum* strain CDC667 containing a 'silent' BoNT/B gene (designated type A (B)). It was found that the NTNH adjacent to the defective BoNT/B gene was 'mosaic', in that the 5′ and 3′-regions of the gene showed high homology (*ca.* 99% amino acid identity) with corresponding regions of proteolytic type B

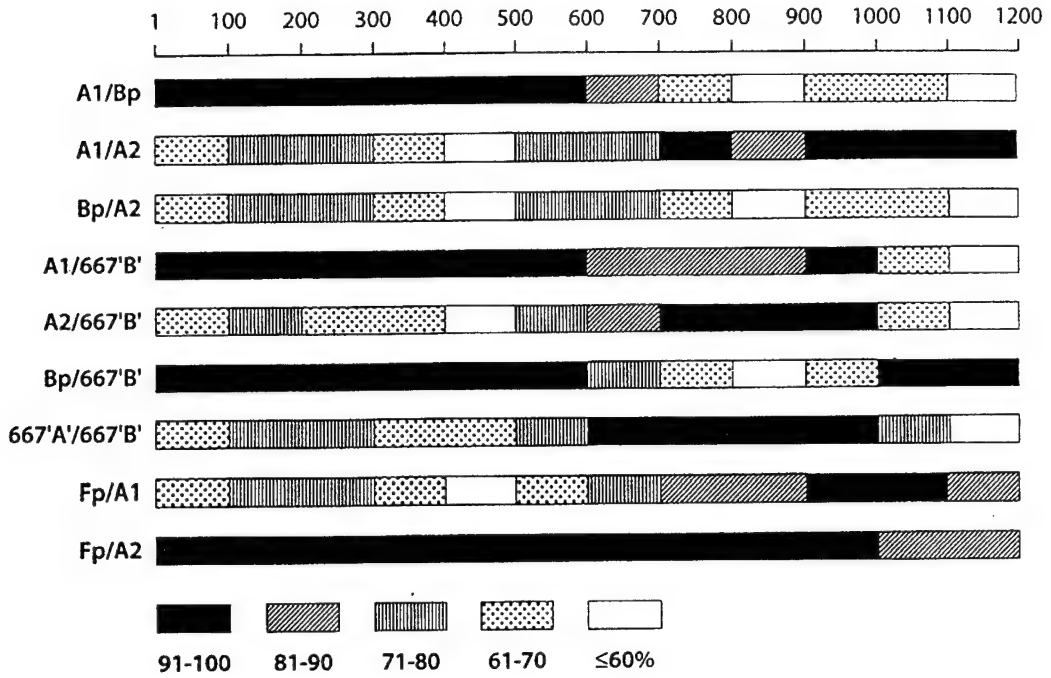


Figure 3. Pairwise analysis showing amino acid identity within the NTNHs encoded by strains of proteolytic *C. botulinum*. For strain CDC667, 667'A' refers to the NTNH encoded upstream of BoNT/A gene and 667'B' refers to that upstream of the silent BoNT/B gene. Other strains: A1 = 62A; Bp = NCTC 7273; A2 = infant type A Kyoto-F; Fp = Langeland.

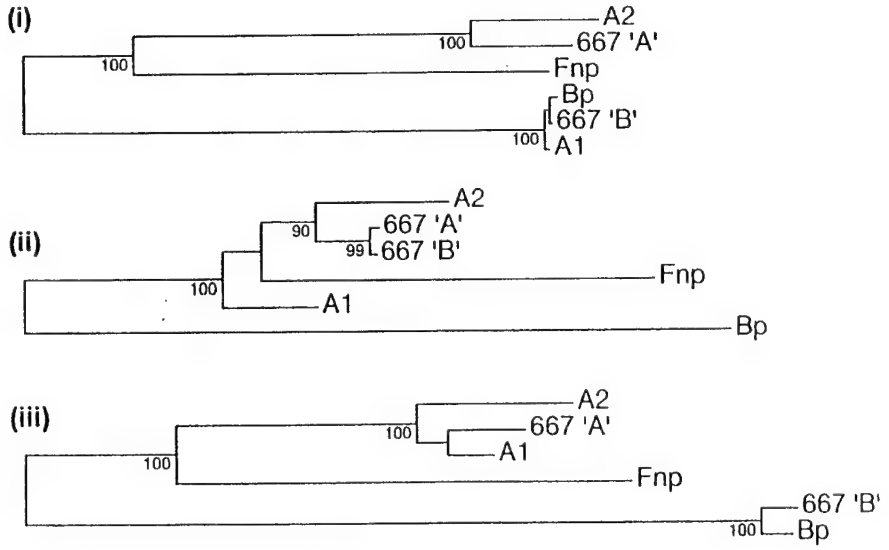


Figure 4. Trees revealing the discordance in relationships along the length of NTNHs: (i) residues 1-550, (ii) residues 551-1020, (iii) residues 1021-C-terminus. NTNH sequences from the following were compared: proteolytic *C. botulinum* type A strains 62A (A1) and Kyoto-F (A2), type B strain NCTC 7273 (Bp), type A(B) strain 667: 'A' cluster (667'A') and 'B' cluster (667'B'), and non-proteolytic *C. botulinum* type F strain Eklund 202F (Fnp). The numbers on the tree indicate bootstrap values for branch points, only values of >90 are shown.

NTNH, while a central region of about 470 amino acids was identical with the equivalent region of the NTNH encoded in the type A gene cluster. A subsequent study by East *et al.* (1996) has shown such 'chimera-like' NTNH genes are common-place within group I *C. botulinum* organisms. Figure 3 shows examples of pairwise analyses performed on NTNH sequences of proteolytic *C. botulinum* strains and illustrates that for some pairs of sequences there is considerable variation in the degree of homology along their lengths. Figure 4 depicts genealogical trees constructed from different regions of the NTNH and reinforces the incongruence in the pattern of observed relationships along the length of the NTNHs. Such marked discordance could be indicative of recombination and suggest the NTNH gene may be a 'hot spot' for such events within the BoNT complex cluster of genes. However, irrespective of the possible mechanisms it is evident that the genealogy of the NTNHs does not mirror that of the BoNTs. Clearly much more comparative sequence data is required on a large number of strains (including non-botulinum species such as *C. baratii*) and on the full complement of associated genes before a thorough understanding of the phylogenetics of the botulinum neurotoxins and its non-toxic complex proteins is to be realized.

References

Aureli P Fenecia L Pasolini B Bianfranceschi M McCroskey LM and Hatheway CL (1986) Two cases of type E infant botulism caused by neurotoxigenic *Clostridium butyricum* in Italy *J Infect Dis* **15**, 207-211.

Collins MD Lawson PA Willems A Cordoba JJ Fernandez-Garaysabal J Garcia P Cai J Hippe H and Farrow JAE (1994) The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations *Int J System Bacteriol* **44**, 812-826.

East AK Bhandari M Stacey JM Campbell KD and Collins MD (1996) Organisation of the cluster of genes encoding components of the botulinum toxin complex in proteolytic *Clostridium botulinum* types A, B and F: evidence of chimeric sequences in the gene encoding the nontoxic-nonhemagglutinin *Int J System Bacteriol* **46**, 1105–1112.

Elmore MJ Hutson RA Collins MD Bodsworth NJ Whelan SM and Minton NP (1995) Nucleotide sequence of the gene coding for proteolytic (group I) *Clostridium botulinum* type F neurotoxin: genealogical comparison with other clostridial neurotoxins *Syst Appl Microbiol* **18**, 23-31.

Gimenez DF and Ciccarelli AS (1970) Another type of *Clostridium botulinum Zentralbl Bakteriol Parasitenkd Infektionskr Abt Orig Reihe A* **215**, 215-220.

Hall JD McCrosley L Pincomb BJ and CL Hatheway (1985) Isolation of an organism resembling *Clostridium barati* which produces type F botulinum toxin from an infant with botulism *J Clin Microbiol* **21**, 654–655.

Hatheway CL (1990) Toxigenic clostridia *Clin Microbiol Rev* **3**, 66-98.

Hatheway CL (1992) *Clostridium botulinum* and other clostridia that produce botulinum neurotoxin In Hauschild AHW and Dodds KL (eds) *Clostridium botulinum – Ecology and Control in foods*. New York: Marcel Dekker Inc. pp 3–20.

Holdeman LV and Brooks JB (1970) Variation among strains of *Clostridium botulinum* and related clostridia In Herzberg M (ed.) Proceedings of the first U.S. – Japan conference of toxic microorganisms. Washington D.C: U.S. Government Printing Office, pp 278–286.

Hutson RA Thompson DE and Collins MD (1993a) Genetic interrelationships of asaccharolytic *Clostridium botulinum* types B, E and F and related clostridia as revealed by small-subunit rRNA gene sequences *FEMS Microbiol Lett* **108**, 103–110.

Hutson RA Thompson DE Lawson PA Schocken-Itturino RP Böttger EC and Collins MD (1993b) Genetic interrelationships of proteolytic *Clostridium botulinum* types A, B, and F and other members of the *Clostridium botulinum* complex as revealed by small-subunit rRNA gene sequences *Antonie van Leeuwenhoek* **64**, 273–283.

Hutson RA Zhou YK Collins MD Johnson EA Hatheway CL and Sugiyama H (1996) Genetic characterization of *Clostridium botulinum* type A containing silent type B neurotoxin gene sequences *J Biol Chem* **271**, 10786–10792.

McCroskey LM Hatheway CL Fenicia L Pasolini B and Aureli P (1986) Characterization of an organism that produces type E botulinal toxin but which resembles *Clostridium botulinum* from the feces of an infant with type E botulism *J Clin Microbiol* **23**, 201-202.

McCroskey LM Hatheway CL Woodruff BA Greenberg JA and Jurgenson P (1991) Type F botulism due to neurotoxigenic *Clostridium barati* from an unknown source in an adult *J Clin Microbiol* **29**, 2618-2620.

Prevot AR (1953) Rappor d' introduction du president du sous-comite *Clostridium* pour l'unification de la nomenclature des types toxigeniques de *C. botulinum Int Bull Bacteriol Nomencl* **3**, 120-123.

Smith LDS and Hobbs G (1974) Genus III *Clostridium* Prazmowski 1880 23 In Buchanan RE and Gibbons NE (eds) Bergey's manual of determinative bacteriology, 8th edn. Baltimore: The Williams and Wilkins Co. pp 551-572.

Session 3

Structure, Action and as Probes I

The metalloproteinase activity of clostridial neurotoxins

Rossella Pellizari[1], Fiorella Tonello[1], Philip Washbourne[1], Silvia Morante[2], Ornella Rosetto[1], Giampietro Schiavo[1] and Cesare Montecucco[1]

[1]Centro CNR Biomembrane and Dipartimento di Scienze Biomediche, Universita', di Padova, Via Trieste 75, 35121 Padova, Italy and [2]Dipartimento di Fisica, Università di Roma a Tor Vergata, Roma, Italy

Keywords: tetanus, botulism, neurotoxins, zinc, exocytosis, SNARE, synaptic vesicle

Clostridial neurotoxins form a novel group of metalloproteinases with specific structural and functional characteristics. Metal substitution and spectroscopic investigations of tetanus neurotoxin indicate that its active site is different from those of the astacin family and of the thermolysin family of metalloproteinases. Immunochemical, biochemical and site-directed mutagenesis investigations of the neurotoxins highlighting the molecular basis of their specific recognition of three SNARE proteins are presented. They indicate that major determinants of such a specific interaction are two non contiguous regions of the substrates: **a)** the cleavage site sequence and **b)** a SNARE motif present in each of the toxin-sensitive SNARE proteins. Other regions may be involved in modulating more specific interactions between each neurotoxin-substrate complex.

Introduction

Several structural and functional features identify tetanus and botulinum neurotoxins to be a distinct group of zinc-endopeptidases. They have a unique three-domain structure. They are composed of two disulfide-linked polypeptide chains. H (100 kDa) consists of two 50 kDa domains, each of which is implicated in a distinct step of the neuron intoxication process. The carboxyl-terminal domain, termed H_C, is involved in neurospecific binding to the presynaptic membrane, whereas the amino-terminal domain is thought to be responsible for the translocation of the enzymic L chain (50 kDa) in the cytosol. The interchain disulfide bond has to be reduced in order to free the proteolytic activity of the L chain. This reduction normally takes place inside the cell. Hence, these proteinases are inactive outside cells contrasting with most proteolytic enzymes which are active outside cells. The L chains of the eight clostridial neurotoxins are very similar suggesting that they derive from a common ancestor. The most conserved segment is located in the central part of the L chain and includes the HExxH zinc binding motif. However, apart from this motif, this central segment has no similarity to any other known zinc-endopeptidase. They are remarkably specific. They only cleave one protein substrate at a single peptide bond. TeNT and BoNT/B, /D, /F and /G act only on VAMP. BoNT/A and /E are very specific for SNAP-25. The only exception is BoNT/C which recognizes and cleaves two protein substrates: SNAP-25 and syntaxin. They do not cleave short peptides, derived from the substrates, encompassing the known cleavage site. Only one peptide bond of a given chemical composition, out of several present in the substrate molecule, is cleaved. BoNT/C only cleaves syntaxin and SNAP-25 when they are bound to the membrane (for a discussion of these points see Niemann *et al.*, 1994; Montecucco and Schiavo, 1995). These peculiar features not only identify the clostridial neurotoxins as an independent group of metalloproteinases (Montecucco and Schiavo, 1993), but also indicate that they recognize the three-dimensional structure of their three proteolytic substrates.

Here, novel experimental evidence that further characterize this group of enzymes is presented together with data that provide novel insights into the molecular basis of the specific recognition of the eight neurotoxins for their substrates.

To whom correspondence should be sent: C. Montecucco
Dipartimento Science Biomediche, Via Trieste, 75, 35121, Padova, Italy.
tel. +39.49.8276069; fax +39.49.8276049
e.mail: venom@civ.bio.unipd.it

Zinc co-ordination in clostridial neurotoxins

Information on zinc co-ordination in proteins can be obtained by analyzing the effect of substituting the

zinc atom with other transition metal ions such as cobalt (II), copper (II), nickel (II), iron (II), manganese (II). In particular, cobalt is a sensitive probe of the metal environment because its derivatives absorb light in the visible range. Co(II) binds to thermolysin via two histidines and a glutamate and its visible adsorption spectrum shows a maximum at 550 nm (Matthews *et al.*, 1972; Holmquist and Valle, 1974). Astacin, the prototype of a large group of metalloproteinases, binds Co^{2+} via the two histidines of the HExxH motif, an additional histidine and a tyrosine residue and shows a maximum at 514 nm (Bode *et al.*, 1992). The zinc atom of TeNT was removed by incubating the toxin with ortho-phenanthroline (Stocker *et al.*, 1988; Gomis-Ruth *et al.*, 1994). The inactive apo-TeNT and the holo-toxin can be reformed by incubation with zinc- containing buffers. The addition of different metal ions was achieved by dialysis of the apo-TeNT with equimolar solutions of the appropriate metal ion, followed by extensive dialysis with metal-free buffer solutions. Different metal ions associate with TeNT to different final stoichiometries: as expected zinc re-associated with higher efficiency, followed by the other ions in the order $Zn^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+} > Fe^{2+}$. This order of efficiency of metal ion binding to apo-TeNT is different from that found with thermolysin ($Zn^{2+} > Co^{2+} > Mn^{2+} > Fe^{2+} > Ni^{2+}$) (Holmquist and Valle, 1974) and astacin ($Zn^{2+} > Fe^{2+} = Cd^{2+} > Co^{2+} > Ni^{2+} > Cu^{2+} > Mn^{2+}$) (Stocker *et al.*, 1988). The proteolytic activity of the metal-substituted TeNT preparations was determined by using as a substrate metal-free recombinant GST-VAMP-2 and the data were corrected for the actual amount of holo-TeNT. The following order of proteolytic activity was obtained: $Mn^{2+} > Zn^{2+} = Co^{2+} > Ni^{2+} > Fe^{2+}$. Such order is different from that of thermolysin ($Co^{2+} > Zn^{2+} > Fe^{2+} > Mn^{2+} > Ni^{2+}$ (Holmquist and Valle, 1974) and of astacin ($Co^{2+} > Zn^{2+} > Cu^{2+} > Hg^{2+} > Ni^{2+}$) (Stocker *et al.*, 1988; Gomis-Ruth *et al.*, 1994). These results clearly indicate that the zinc environment at the active site of TeNT is different from those of thermolysin and of astacin. Further strong support to this conclusion is provided by the analysis of the visible spectrum of Co-TeNT shown in Fig. 1. This spectrum shows a maximum at 535 nm and a shoulder at 480 nm and clearly differs from the absorption spectra of Co-thermolysin and Co-astacin.

Insights into the ligands involved in zinc coordination in TeNT comes from a recently performed X-ray absorption spectroscopy (XAS) comparative study (Morante *et al.*, 1996). XAS can provide information on the type of residues present in the first coordination shell of metal in proteins and the average distance of the metal from binding atoms. Since no

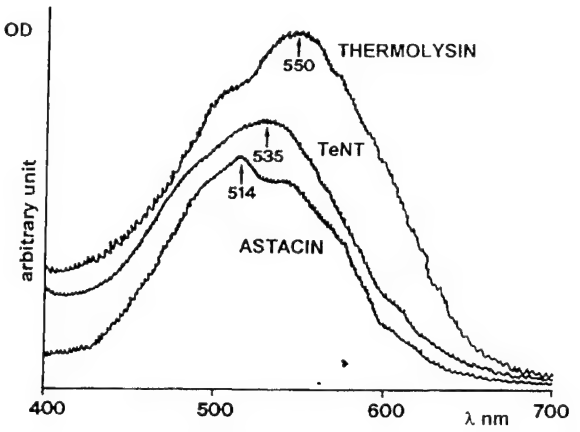


Figure 1 Visible absorption spectra of Co-Thermolysin, Co-Tetanus neurotoxin and Co-Astacin. All spectra were recorded after extensive dialysis of the metal-substituted proteins in a Perkin-Elmer lambda-6 spectrophotometer.

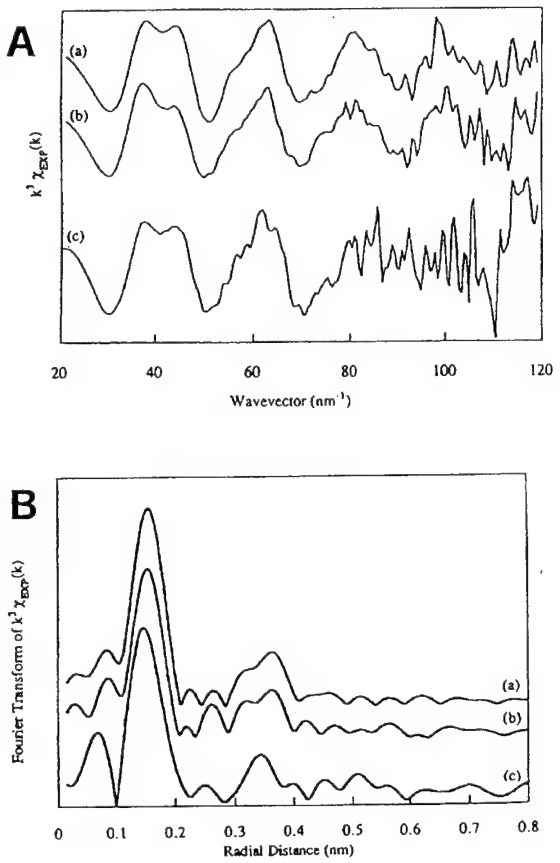


Figure 2 X-ray absorption spectra of tetanus neurotoxin, thermolysin and astacin. **A)** plot of the experimental EXAFS signal; **B)** Fourier transforms of the EXAFS spectra of panel **A.**

XAS spectra for any known zinc-endopeptidases has been reported, this study was extended for comparison to include thermolysin and astacin, whose high resolution crystallographic structure is available (Matthews *et al.*, 1972; Bode *et al.*, 1992). Fig. 2 compares the Zn K-edge XAS spectra and the Fourier transforms of the EXFAS spectra TeNT with those of astacin and thermolysin. These results are analysed in detail elsewhere (Morante *et al.*, 1996) and indicate that groups of similar chemical nature are located around the zinc atom in TeNT and astacin and that they differ from thermolysin. In particular, it should be noted that in the Fourier transformed spectra the most visible difference between thermolysin and the other three samples is in the shape of the peak structure around 3.5 A. This peak is normally attributed to the residual multiple scattering effect due to the presence of aromatic rings. The main difference in terms of aromatic residues present around the zinc atom between thermolysin and astacin is the presence of an additional histidine and of the distal tyrosine. Thus, these data suggest that around the zinc atom of TeNT there are three, possibly four, aromatic residues. Since a previous study had shown that two histidines are involved in zinc coordination in TeNT (Schiavo *et*

```
TeNT     D P A L L L M H E L I H V L H G L Y G

BoNT/A   D P A V T L A H E L I H A G H R L Y G
BoNT/B   D P A L I L M H E L I H V L H G L Y G
BoNT/C   D P I L I L M H E L N H A M H N L Y G
BoNT/D   D P V I A L M H E L I H S L H Q L Y G
BoNT/E   D P A L T L M H E L I H S L H G L Y G
BoNT/F   D P A I S L A H E L I H A L H G L Y G
BoNT/G   D P A L T L M H E L I H V L H G L Y G
         * *       *   * * *   *     *   * * *
                     * * *   *

Astacin  V Y H G T I I H E L M H A I G F Y H E
```

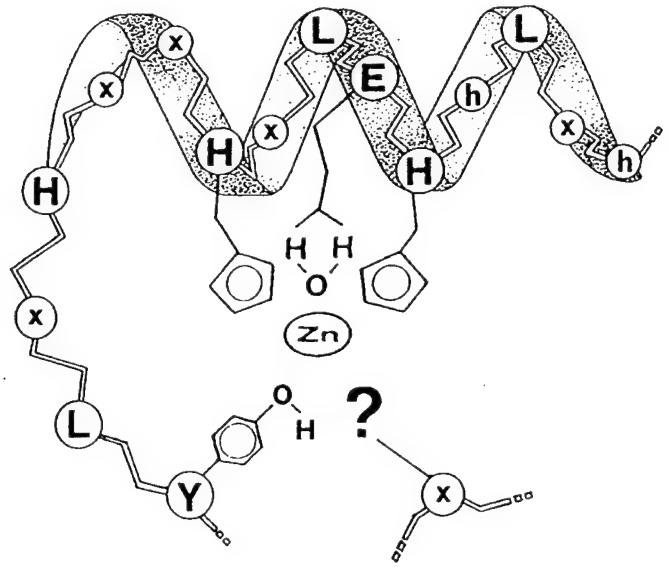


Figure 3 Zinc coordination by the L chain of clostridial neurotoxin. The upper panel reports the sequence of the highly conserved central portion of the L chains of tetanus and botulinum neurotoxins, together with that of the astacin family of metallo-proteinases. The lower panel depicts the proposed mode of zinc coordination at the active site of clostridial neurotoxins, based on sequence comparison and spectroscopic data. Whereas the involvement of the two imidazole groups and the water molecule is well established, that of the conserved tyrosine present in the segment and of a possible additional aromatic residue is to be definitively proven. Notice that the suggested tyrosine fourth ligand of clostridial neurotoxins occupies the same positions as the histidine fourth zinc ligand of astacin-like proteinases.

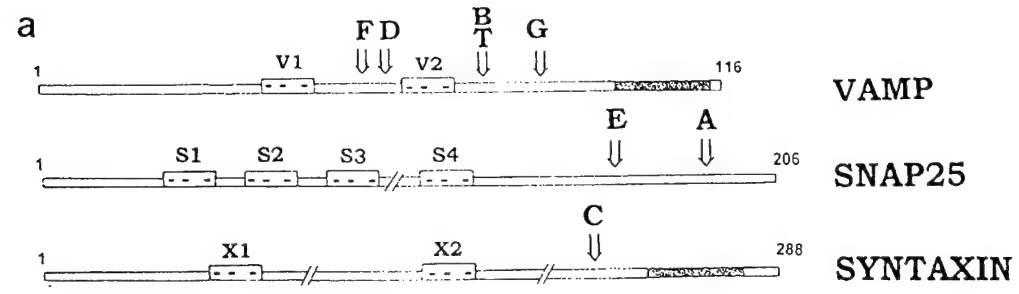
al., 1992a), this spectroscopic result can be accounted for by the presence of a tyrosine. These studies cannot be extended to the botulinum neurotoxins because of the high amounts of protein needed. However, sequence comparison may help to extend information to the entire group of clostridial neurotoxin metallo-proteinases. Fig. 3 compares the sequences of the conserved central segment of the L chains of clostridial neurotoxins, including the zinc binding motif, with that of the astacin family of zinc-endopeptidases. The third histidine ligand of astacin is present in a highly conserved region and includes a glycine residue which bends the polypeptide chain to bring the third histidine close to the zinc atom. This glycine residue is not present in the clostridial neurotoxins, where it is replaced by a conserved histidine, mutation of which does not alter proteolytic activity (Yamasaki *et al.*, 1994a). Clostridial neurotoxins have a conserved tyrosine at the position of the third astacin histidine and its replacement with a phenyl alanine residue inactivates TeNT (Yamasaki *et al.*, 1994a). All together these results indicate that tyrosine-242 is a zinc ligand in TeNT, as shown in Fig. 3 B. Tyrosine-242 is brought next to the zinc atom by a bend of the polypeptide chain, most likely at the level of the histidine-239 and glycine-240 residues. These residues are conserved among most clostridial neurotoxins or are substituted by residues compatible with a polypeptide chain bending.

The specific recognition of VAMP, SNAP-25 and syntaxin by clostridial neurotoxins

The only known proteolytic substrates of the clostridial metallo-proteinases are the three SNARE (soluble NSF accessory protein receptors) proteins: VAMP, SNAP-25 and syntaxin. Several studies have documented that short peptides encompassing the cleavage site are not cleaved although they bind the toxin (Schiavo *et al.*, 1992 ab; Shone *et al.*, 1993) and that only long segments of the substrate sequence are cleaved (Shone *et al.*, 1993; Foran *et al.*, 1994; Cornille *et al.*, 1994, Yamasaki *et al.*, 1994b). Although TeNT and BoNT/B hydrolyse the same peptide bond of VAMP, BoNT/B cleaves peptide 44-94 of VAMP, whereas TeNT only cleaves VAMP peptide 33-94 or longer (Foran *et al.*, 1994). Furthermore several neurotoxins hydrolyse a single peptide bond and leave intact other peptide bond(s) of the same type located in another part of the substrate molecule. For example: (i) BoNT/D cleaves rat VAMP-2 at the lysine59-leucine60 peptide bond and leaves intact the bond at lysine83-leucine84 (Schiavo *et al.*, 1993a,); (ii) BoNT/G cleaves rat VAMP-2 at the alanine81-alanine82 peptide bond and leaves intact the alanine5-alanine6 bond (Schiavo *et al.*, 1994; Yamasaki *et al.*, 1994b); (iii) BoNT/A cleaves SNAP-25 at the glutamine197-arginine198 peptide bond and leaves intact the glutamine15-arginine16 bond in the same molecule (Schiavo *et al.*, 1993b; Binz *et al.*, 1994); (iv) BoNT/E cleaves SNAP-25 at the arginine180-isoleucine181 peptide bond but leaves intact the arginine59-isoleucine60 bond of SNAP-25, (Schiavo *et al.*, 1993b; Binz *et al.*, 1994); (v) BoNT/C cleaves syntaxin 1a at the lysine253-alanine254 peptide bond and does not affect the lysine260-alanine261 bond of syntaxin, (Schiavo *et al.*, 1995). Several investigators have further demonstrated that BoNT/C only cleaves membrane-bound SNAP-25 and syntaxin and that other neurotoxins are more effective on the membrane bound substrate than on the recombinant soluble molecule (Blasi *et al.*, 1993; Osen-Sand *et al.*, 1996; Williamson *et al.*, 1996; Pellizzari *et al.*, 1996).

To account for these findings, it was proposed that clostridial neurotoxins recognise the tertiary, rather than the primary, structure of the SNAREs (Rossetto *et al.*, 1994; Foran *et al.*, 1994). Moreover, we identified a motif common to the three SNARE proteins, thereafter called the SNARE motif, and we proposed that this motif is a major determinant of the specificity of clostridial neurotoxins for the three SNARE proteins (Rossetto *et al.*, 1994). As shown in Fig. 4, there are two copies of the motif in VAMP (V1 and V2) and syntaxin (X1 and X2) and four copies in SNAP-25 (S1, S2, S3 and S4). The motif is characterised by the presence of three carboxylate residues and three hydrophobic residues. Several pieces of experimental evidence support this proposal: (a) only those SNARE segments including at least one SNARE motif are cleaved (Shone *et al.*, 1993; Cornille *et al.*, 1994; Foran *et al.*, 1994; Yamasaki *et al.*, 1994b), (b) the motif is exposed on the protein surface as demonstrated by binding of anti-SNARE motif antibodies, (c) these antibodies cross-react among the three SNAREs and inhibit the proteolytic activity of the neurtoxins and (d) the various neurotoxins cross-inhibit each other (Pellizzari *et al.*, 1996).

As a first approach to evaluate the role of the various residues of the motif in toxin binding, the aspartate and glutamate residues of V1 and V2 were replaced respectively by asparagine and glutamine residues in GST-VAMP-2 fusion proteins. The purified recombinant proteins were inserted in asolectin liposomes and the kinetics of hydrolysis catalyzed by TeNT or BoNT/B determined by densitometric scanning of SDS-PAGE gels. Fig. 4 shows that removal of the negative charges from V2 had a profound effect on the proteolytic activity of BoNT/B, but had no effect on that of TeNT. *Vice versa*, the same mutations in V1 had no effect on the susceptibility of VAMP-2 to BoNT/B, but totally abolished cleavage



b

V1	^{38}Q	V	D	E	V	V	D	I	M	R ^{47}
V2	^{62}E	L	D	D	R	A	D	A	L	Q ^{71}
S1	^{21}L	A	D	E	S	L	E	S	T	R ^{30}
S2	^{35}L	V	E	E	S	K	D	A	G	I ^{44}
S3	^{49}M	L	D	E	Q	G	E	Q	L	E ^{58}
S4	^{145}E	M	D	E	N	L	E	Q	V	S ^{154}
X1	^{29}F	M	D	E	F	F	E	Q	V	E ^{38}
X2	^{164}E	L	E	D	M	M	L	E	S	G ^{173}

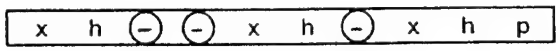


Fig. 4. Neurotoxin recognition motif of VAMP, SNAP-25 and syntaxin. **a)** boxes indicate the positions of a common motif in the three substrates of clostridial neurotoxins. The motif is presented in two copies in VAMR (V1 and V2), two copies in syntaxin (x1 and X2) and four copies in SNAP-25 (S1, S2, S3 and S4); The capital letters correspond to the proteolytic cleavage sites of different clostridial neurotoxin. **b)** sequences of the variuos copies of the SNARE motif.

by TeNT. Hence, two neurotoxins that cleave the same substrate at the same peptide bond appear to recognize it via two different copies of the SNARE motif: V1 is involved in TeNT binding and V2 is implicated in BoNT/B binding. This result explains why the minimal length of VAMP segments cleaved by TeNT is longer than that required by BoNT/B (Shone *et al.*, 1993; Foran *et al.*, 1994), since they have to include V1 which is more amino-terminal with respect to V2. Comparison of the sequence and of the predicted secondary structure of TeNT and BoNT/B indicate that they are closely similar, suggesting that also the spatial distribution of the residues involved in VAMP binding is similar. On this basis and in light of the results of Fig. 4, we would like to propose that the two copies of the

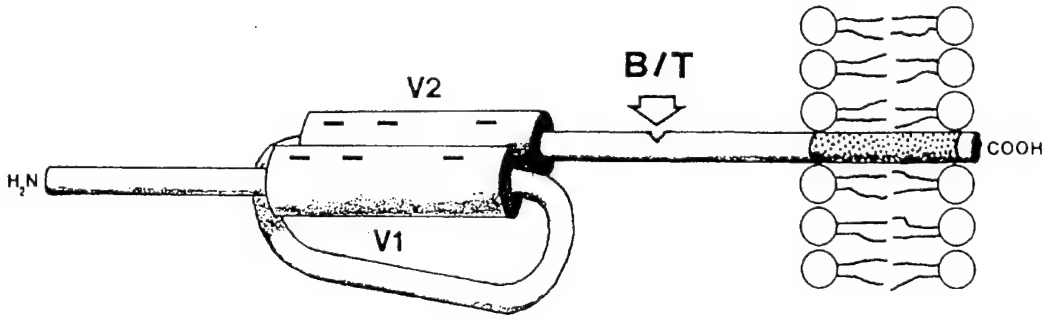


Fig. 5. Proposed tandem arrangement of the two copies of the SNARE motif of VAMP. Such an arrangement is proposed to account for the results of proteolysis of VAMP mutants in V1 and V2 by tetanus and botulinum B neurotoxins (see text)

SNARE motif of VAMP are arranged as a tandem in such a way that they adopt the same spatial orientation with respect to the glutamine76-phenylalanine77 bond, as depicted in Fig. 5. We do not exclude the likely possibility that other regions of VAMP or SNAP-25 or syntaxin are important for maximal interaction among each neurotoxin-SNARE pair. In the case of TeNT and BoNT/B it is clear that the positively charged region towards the carboxy-terminal side of the cleavage site of VAMP is required for maximal cleavage rate (Shone *et al.*, 1993; Yamasaki *et al.*, 1994b). Here, we propose that a major common determinant of the specificity of the clostridial neurotoxins for the three SNARE proteins is based on recognition of the SNARE motif. This is closely followed by specific binding to the segment containing the susceptible peptide bond. The relative contribution of the two interactions to the specificity and strength of neurotoxin binding remains to be determined. It is expected that hydrolysis of the substrate region bound to the active site of the neurotoxin leaves the toxin bound to its substrate predominantly via its interaction with the SNARE motif. This should result in a large decrease in binding affinity, which in turn should lead to a rapid release of the hydrolysed substrate.

Acknowledgments

The work described here was supported by grants n. 473 and 763 of Telethon-Italia and by CNR.

References

Binz T, Blasi J, Yamasaki S, Baumeister A, Link E, Sudhof TC, Jahn R and Niemann H (1994) Proteolysis of SNAP-25 by types E and A botulinal neurotoxins. *J Biol Chem* **269**, 1617-1620.

Blasi J, Chapman ER, Yamasaki S, Binz T, Niemann H and Jahn R(1993) Botulinum neurotoxin C blocks neurotrasmitter release by means of cleaving HPC-I/syntaxin. *EMBO J* **12**, 4821-4828.

Bode W, Gomis-Ruth FX, Huber R, Zwilling R and Stocker W (1992) Structure of astacin and implication of astacins and zinc-ligation of collagenases. *Nature* **358**, 164-166.

Cornille F, Goudreau N, Ficheux D, Niemann H and Roques BP (1994) Solid phase synthesis, conformational analysis and in vitro cleavage of synthetic himan synaptobrevin II 1-93by tetanus toxin L chain. *Eur J Biochem* **222**, 173-181.

Foran P, Shone CC and Dolly JO (1994) Differences in the protease activities of tetanus and botulinum B toxins revealed by the cleavage of vesicle-associated membrane protein and various sized fragments. *Biochemistry*, **31**, 15365-15374.

Gomis-Ruth F, Grams F, Yallouros I, Nar H, Kusthardt U, Zwilling R, Bode W and Stocker W (1994) Crystal structures, spectroscopic features and catalytic properties of Cobalt(II), Copper(II), Nickel(II), and Mercury(II) derivatives of the zinc endopeptidase astacin. *J Biol Chem* **269**, 17111-17117.

Holmquist B and Valle BL (1974) Metal substitution and inhibition of thermolysin: spectra of the cobalt enzyme. *J Biol Chem* **249**, 4601-4607

Matthews BW, Jansonius JN and Colman PM (1972) Three-dimensional structure of thermolysin. *Nature New Biol* **238**, 37-41.

Montecucco C and Schiavo G (1993) Tetanus and botulism neurotoxins: a new group of zinc proteases. *Trends Biochem Sci* **18**, 324-327.

Montecucco C and Schiavo G (1995) Structure and function of tetanus and botulinum neurotoxins. *Quart Rew Biophys* **28**, 423-472.

Morante S, Furenlid L, Schiavo G, Tonello F, Zwilling R. and Montecucco C, (1996) X-ray absorpion spectroscopy study of zinc coordination in tetanus neurotoxin, astacin, alkaline protease and thermolysin. *Eur J Biochem* **235**, 606-612.

Niemann H, Blasi J and Jahn R (1994) Clostridial neurotoxins: new tools for dissecting exocytosis. *Trends Cell. Biol* **4**, 179-185.

Osen-Sand A, Staple JK, Naldi E, Schiavo G, Rossetto O, Petit-pierre S, Malgaroli A, Montecucco C and Catsicas S (1996) Common and distinct fusion proteins in axonal growth and trasmitter release. *J Comp Neurol* **367**, 222-234.

Pellizzari R, Rossetto O, Lozzi L, Giovedì S, Johonson E, Shone CC and Montecucco C (1996) Structural determinants of the specificity for VAMP/synaptobrevin of tetanus and botulinum B and G neurotoxins. *J Biol Chem In press.*

Rossetto O, Schiavo G, Montecucco C, Poulain B, Deloy F, Lozzi L and Shone C. C (1994) SNARE motif and neurotoxin recognition. *Nature* **372**, 415-416.

Schiavo G, Poulain B, Rossetto O, Benfenati F, Tauc L. and Montecucco C (1992a) Tetanus toxin is a zinc protein and its inhibition of neurotrasmitter release and protease activity depend on zinc. *EMBO J* **11**, 3577-3583.

Schiavo G, Rossetto O, Santucci A, DasGupta BR and Montecucco C (1992b) Botulinum neurotoxins are zinc proteins. *J Biol Chem* **267**, 23479-23483.

Schiavo, G, Rossetto O, Catsicas S, Poleverino De Laureto P, DasGupta BR, Benfenati F and Montecucco C(1993a) Identification of the nerve-terminal targets of botulinum neurotoxins serotypes A, D and E. *J Biol Chem* **268**, 23784-23787.

Schiavo G, Santucci A, DAsGupta BR, Metha PP, Jontes J, Benfenati F, Wilson MC and Montecucco C (1993b) Botulinum neurotoxins serotype A and E cleave SNAP-25 at distinct COOH-terminal peptide bonds. *FEBS Lett* **335**, 99-103.

Schiavo G, Malizio C, Trimble WS, Polverino De Laureto P, Milan G, Sugiyama H, Johnson EA and Montecucco C (1994) Botulinum G neurotoxin cleaves VAMP/synaptobrevin at a single Ala/Ala peptide bonds. *J Biol Chem* **269**, 20213-20216.

Schiavo G, Shone CC, Bennet MK, Scheller RH and Montecucco C (1995) Botulnim neurotoxin type C cleaves a single Lys-Ala bond within the carboxyl-terminal region of syntaxins. *J Biol Chem* **270**, 10566-10570.

Shone CC, Quinn CP, Wait R, Hallis B, Fooks SG and Hambleton P (1993) Proteolytic cleavage of synthetic fragments of vesicle-associated-membrane protein, isoform-2 by botulinum type B neurotoxin. *Eur J Biochem* **225**, 263-270.

Stocker W, Wolz RL, Zwilling R, Strydom DJ. and Auld DS (1988) Astacus Protease, a zinc metalloenzyme. *Biochemistry* **27**, 5026-5632.;

Williamson L, Halpern JL, Montecucco C, Brown JE and Neale EA (1996) Clostridial neurotoxins and substrate proteolysis in intact neurons. *J Biol Chem* **271**, 7694-7699.

Yamasaki S, Hu Y, Binz T, Kalkuhl A, Kurazono H, Tamura T, Jahn R, Kandel E and Niemann H (1994a). Synaptobrevin/VAMP of *Aplysia californica*: structure and proteolysis by tetanus and botulinal neurotoxins type D and F. *Proc Natl Acad Sci USA* **91**, 4688-4692.

Yamasaki S, Baumeister A, Binz T, Blasi J, Link E, Cornille F, Roques B, Fykse EM, Sudhof TC, Jahn R and Niemann H (1994b) Cleavage of members of synaptobrevin/VAMP family by types D and F botulinal neurotoxins and tetanus toxin. *J Biol Chem* **269**, 12764-12772.

Study of specificity of the endopeptidase activities of the botulinum neurotoxins using peptide substrates

Matthew Wictome[1], Ornella Rossetto[2], Cesare Montecucco[2] and Clifford C. Shone[1]

[1]Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire, SP4 0JG, UK.
[2]Dipartimento di Scienze Biomediche, Universita di Padova, Via Trieste 75, Padova, Italy

Each of the *Clostridium botulinum* neurotoxins (BoNTs) exerts its intracellular activity via highly specific, zinc-dependent endopeptidase activities which cleave small proteins (SNARES) involved in vesicle docking/fusion. The precise nature of these highly specific enzyme-substrate interactions has yet to be elucidated but determinants other than the cleavage site amino acid sequence appear to be involved. Recently, it has been suggested that a nine residue motif, of which there are multiple copies in VAMP, SNAP-25 and syntaxin, acts as common recognition sites for the BoNTs. This 'sub-site', in conjunction with the cleavage site, may form the basis of a dual recognition mechanism by the toxins for their substrates. Evidence in favour of such a mechanism has been obtained from studies of the endopeptidase activity of BoNT/B using VAMP peptide substrates. Aspartate residues within the motif which flank the N-terminal side of the cleavage site were found to be essential determinants for the endopeptidase activity of BoNT/B. Studies on the specificity of BoNT/A and BoNT/E were less conclusive. Both of the latter toxins were found to cleave SNAP-25 peptides which lack motif sequences.

The BoNTs act on the peripheral nervous system where they inhibit the release of acetylcholine at the neuromuscular junction, an action which results in widespread paralysis and ultimately the syndrome botulism. In their activated forms, the clostridial neurotoxins consist of two subunits; a heavy chain (100 kDa) and a light chain (50 kDa), linked by a disulphide bridge (Niemann, 1991). All of the BoNTs have been shown to mediate this intracellular action via highly specific zinc-endopeptidase activities contained within the light subunit of each toxin (Schiavo *et al.*, 1995). Collectively, the clostridial neurotoxins are known to act on various isoforms of just three different proteins which form part of a putative vesicle docking/fusion mechanism (Sollner *et al.*, 1993). BoNT/B, D, F and G cleave vesicle-associated membrane protein (VAMP or synaptobrevin), at different sites. BoNT/A and E cleave the protein SNAP-25 at distinct sites close to the C-terminus and BoNT/C_1 toxin cleaves both syntaxin and SNAP-25.

The mechanism of the enzyme-substrate recognition is presently unclear. Studies using both synthetic peptides and recombinant fragments derived from the target proteins of the toxins have shown that only relatively large peptide fragments are substrates for the toxin zinc-endopeptidases. In the case of BoNT/B only VAMP peptide substrates of >30 residues were cleaved by the toxin (Shone *et al.*, 1993). Even larger substrates (>50 residues) are required by tetanus toxin and BoNT/A neurotoxin. Surprisingly, in a study of the peptide substrate specificity of BoNT/B only one VAMP residue at the cleavage site (Phe77) was found to be critical to the endopeptidase activity of the toxin (Shone and Roberts, 1994). These data suggest that determinants other than the cleavage site sequence are required to account for the neurotoxin's highly specific proteolytic activity. In the present study, peptide substrates were used to determine the role of residues flanking the cleavage sites of the BoNTs.

Corresponding author: Dr Clifford C. Shone, CAMR, Porton Down, Salisbury, Wilts SP4 0JG, UK. Telephone: +44 1980 612793; FAX: +44 1980 610848.

Materials and Methods

Purification of Botulinum Type A, B and E Neurotoxins. BoNT/A was purified from the NCTC 7272 strain, BoNT/B from the Okra strain and BoNT/E from the strain NCTC 11219 as described previously (Shone and Tranter, 1995). Purified neurotoxins were

dialysed against 0.05M HEPES buffer pH 7.4 containing 0.15M NaCl and stored at –80 °C.

Synthesis of Peptides. Peptides were synthesised on 4-(2',4'-dimethyl-Fmoc-aminomethyl)-phenoxy resin (Novabiochem) with an automated, solid-phase peptide synthesiser (model 431A, Applied Biosystems Inc, Foster City, CA.) using Perkin-Elmer FastMoc™ chemistry. Peptides were purified by reverse-phase high pressure liquid chromatography on C8 columns (2.2 × 25 cm, Vydac), and characterised as described previously (Shone and Roberts, 1994).

Cleavage of peptides by BoNTs. Peptides (0.31 mM) in 50 mM HEPES buffer pH 7.2 containing 10 μM $ZnCl_2$ and 10 mM 2-mercaptoethanol were incubated at 37 °C for 20 min. BoNTs were diluted with 50 mM HEPES buffer pH 7.2 containing 10 mM dithiothreitol and 20 μM $ZnCl_2$ and incubated at 37 °C for 20 min. Reactions were initiated by addition of 10 μl of toxin solution to the 250 μl peptide mixture and then incubated at 37 °C for 30 min. Proteolysis was stopped by the addition of 0.5 ml of 0.5% (v/v) trifluoroacetic acid. Final concentrations of BoNTs were between 3 and 100 nM and were chosen so that less than 15% of the total peptide was cleaved during the reaction time. The extent of peptide cleavage and initial enzyme rates were estimated from HPLC analysis of reaction mixtures on C8 columns (4.6 × 45 mm, Ultrasphere™, Beckman) as described (Shone and Roberts, 1994).

Results and Discussion

Endopeptidase specificity of BoNT/B

The endopeptidase activity of BoNT/B was studied with either VAMP (60–94) or VAMP (62–91) synthetic peptides derived from the sequence of human VAMP isoform-2. These peptides are cleaved by BoNT/B at the Gln76–Phe77 bond, consistent with *in vivo* observations and at the same rate as larger fragments of VAMP, making them convenient model substrates with which to study the endopeptidase activity of BoNT/B (Shone *et al.*, 1993; Shone and Roberts, 1994).

Table 1 illustrates the effect of neutralisation of various charged residues within VAMP (60–94) peptides on the cleavage rate of BoNT/B. While substitution of Glu62 with a serine residue had no effect on the cleavage rate, similar changes to either Asp64 or Asp65 reduced the cleavage rate to approximately 30–40% of the control value. Substitution or removal of both these aspartate residues virtually abolished cleavage of the peptide by BoNT/B. Analogous substitutions to the positively charged residues to the C-terminal side of the cleavage site also reduced the cleavage rate by BoNT/B but to a

TABLE 1. Influence of charged residues on the cleavage of VAMP peptides by BoNT/B. Substituted residues are shown underlined. The cleavage site for BoNT/B is indicated by italic letters. A putative motif region (residues 62–70) is shown as bold characters in the first sequence.

VAMP Peptide Sequence	Cleavage Rate (nmole sec^{-1})	% Control
(a) Influence of substitutions to negatively charged residues (X = VAMP 78,94)		
60 77 LSE**LDDRADAL**QAGASQ*F* -**X**	24.5 ± 1.5	100%
LSSLDDRADALQAGASQ*F* -**X**	27.2 ± 0.7	111%
LSSLSDRADALQAGASQ*F* -**X**	9.8 ± 1.5	40%
LSSLSSRADALQAGASQ*F* -**X**	0.8 ± 0.05	3.3%
LSELSSRADALQAGASQ*F* -**X**	0.9 ± 0.2	3.7%
RADALQAGASQ*F* -**X**	< 0.5	< 3%
LSELDDRPDALQAGASQF-**X**	17.4 ± 2.5	72%
(b) Influence of substitutions to positively charged residues. **X** = VAMP (60–75)		
76 94 **X**-Q*F*ETSAAKLKRKYWWKNLK	27.3 ± 2.5	100%
X-Q*F*ETSAAKLKRKYWWSNLS	24.8 ± 2.1	91%
X-Q*F*ETSAAKLSSKYWWKNLK	9.0 ± 0.8	33%
X-Q*F*ETSAASLSRKYWWKNLK	7.6 ± 0.5	27.6%
X-Q*F*ETSAAKLSASYWWKNLK	5.5 ± 0.4	20.2%

Table 2. Influence of residue deletions/additions on the cleavage of VAMP peptides by BoNT/B. Amino acid deletions are indicated by a hyphen; additions are shown underlined. The cleavage site for BoNT/B is italic type. Concentrations of BoNT/B were 10nM in (a) and 3.3nM in (b)

VAMP Peptide Sequence	Cleavage Rate (nmole sec^{-1})	% Control
(a) Deletions/additions to the N-terminal region. **X**= VAMP (78,94)		
62 77		
ELDDRADALQAGAS*QF*-**X**	23.4 ± 1.5	100%
ELDDRAD–LQAGAS*QF*-**X**	2.9 ± 0.3	12.3%
ELDDRADALQGAS*QF*-**X**	< 0.5	< 2%
ELDDRADALQ<u>A</u>AGAS*QF*-**X**	< 0.5	< 2%
(b) Deletions to the C-terminal region. **X** = VAMP (62–75)		
76 94		
X-*QF*ETSAAKLKRKYWWKNLK	13.4 ± 1.6	100%
X-*QF*ETSA–KLKRKYWWKNLK	15.5 ± 1.9	116%
X-*QF*ETSAAK–KRKYWWKNLK	17.2 ± 2.1	128%

lesser extent (Table 1). These data suggest that the negatively charged residues, Asp64 and Asp65, N-terminal to the cleavage site of BoNT/B, play a key role in enzyme-substrate recognition.

If Asp64, 65 play a direct role in binding to BoNT/B then their relative position with respect to the cleavage site may be critical. To assess the importance of the relative positions of these residues, the effect of the addition and deletion of amino acid residues was studied. Deletion of either Ala69 or Ala72 greatly reduced the cleavage rate by BoNT/B (Table 2). Addition of an alanine residue at position 72 was found to completely abolish peptide cleavage by BoNT/B (Table 2). Analogous experiments were performed to investigate the role of deleting residues in the peptide sequence flanking the C-terminal side of the cleavage site. In contrast to the above, deletion of either Ala82 or Leu84 were both found to result in a slight increase in the peptide cleavage rate by BoNT/B (Table 2b).

Substitution of Ala67 with a proline residue did not significantly reduce the cleavage rate by BoNT/B which suggests that this region does not have to adopt a helical structure as a prerequisite for cleavage by BoNT/B.

Collectively the above data support the hypothesis of a sub-site for substrate binding in the case of BoNT/B in which the negatively charged residues, Asp64 and 65 play a key role. Either neutralisation of the charges on these residues or change to their relative position with respect to the BoNT/B-cleavage site was found to greatly reduce the rate of proteolysis by the neurotoxin. Both Asp64 and 65 lie within a conserved motif region which has implicated as a recognition site for BoNT/B (Rossetto *et al.*, 1994). The present data fully support the hypothesis that the VAMP motif region (residues 62–70) plays a role in determining the specificity of BoNT/B (Wictome *et al.*, 1996).

Endopeptidase specificity of BoNT/A and BoNT/E

The endopeptidase activities of BoNT/A and BoNT/E were assessed using a range of peptide fragments derived from the COOH-terminal sequence of SNAP-25. The relative cleavage rates are shown in Table 3. Highest rates of cleavage with BoNT/A were observed with SNAP-25 peptides of >60 residues: virtually no endopeptidase activity was observed with peptides of <45 residues. A similar profile of endopeptidase activity was observed with BoNT/E with the exception that cleavage of the 52 residue peptide was relatively higher compared to that of the larger peptides. Notably, both BoNT/A and BoNT/E were found to cleave a 52 residue, SNAP 25 (255–206) peptide which does not contain a copy of the common motif region (Rossetto *et al.*, 1994). One interpretation of these data is that the motif region is not involved in enzyme-substrate recognition in the case of BoNT/A and BoNT/E. Another possibility is that the observed rates of cleavage represent sub-optimal rates of activity as a result of recognition of one binding site of a two-site mechanism. Supportive of this possibility is the significantly higher rates of cleavage observed with BoNT/A using recombinant GST-SNAP-25 as a substrate. Cleavage rates with the latter substrate were found to be 10–100 fold higher than that

Table 3. Relative rate of cleavage of COOH-terminal SNAP-25 peptides by BoNT/A and BoNT/E. In reaction mixtures containing 0.3mM SNAP-25 (137–206) peptide, cleavage rates were 35 ± 3 nmole sec^{-1} for BoNT/A (7nM) and 38 ± 5 nmoles sec^{-1} for BoNT/E (50nM).

COOH-Terminal SNAP-25 Peptide	Relative Rate of Cleavage	
	BoNT/A	BoNT/E
70aas (137–206)	100%	100%
62aas (145–206)	98%	102%
52aas (155–206)	24%	89%
45aas (162–206)	< 3%	5%
35aas (175–206)	< 1%	< 1%

observed with the 70 residue SNAP-25 (137–206) fragment. The observed increase in cleavage rate may result from a different conformation being adopted by the full-length SNAP-25 in which both cleavage site and 'sub-site' can be recognised by BoNT/A. The present data, however, do not rule out a single site mechanism in which a specific conformation at the cleavage site is required.

Conclusions

The endopeptidase activity of BoNT/B was found to be dependent on a pair of aspartate residues (64,65) which lie within a putative common SNARE motif region N-terminal to the cleavage site on VAMP. The distance between this motif region and the cleavage site was found to be critical for optimal endopeptidase activity which suggests a dual site enzyme-substrate recognition mechanism. While a similar mechanism may apply to the cleavage of SNAP-25 by BoNT/A and BoNT/E, present data are not conclusive. For all the BoNTs studied, the length of the peptide substrate greatly influenced the rate of cleavage which suggests that substrate conformation is a key factor in determining the specificity of their endopeptidase activities.

References

Niemann H, (1991) Molecular biology of clostridial neurotoxins. In: Alouf JE and Freer JH (eds): *A Sourcebook of Bacterial Protein Toxins*. Academic Press, London; pp 303–348.

Rossetto O, Schiavo G, Montecucco C, Poulain B, Deloye F, Lozzi L and Shone CC (1994) SNARE motif and neurotoxins *Nature* **372**, 415–416.

Schiavo G, Rossetto O, Tonello F and Montecucco C (1995) Intracellular targets and metalloprotease activity of tetanus and botulinum neurotoxins. In: Montecucco, C (ed.): *Clostridial Neurotoxins*. Springer-Verlag, Berlin; pp 257–274.

Shone CC, Quinn CP, Wait R. Hallis B, Fooks SG and Hambleton, P (1993) Proteolytic cleavage of synthetic fragments of VAMP-2 by botulinum type B neurotoxin. *Eur J Biochem* **217**, 965–971.

Shone CC and Roberts AK (1994) Peptide substrate specificity and properties of the zinc-endopeptidase activity of botulinum type B neurotoxin. *Eur J Biochem* **225**, 263–270.

Shone CC and Tranter HS (1995) Growth of clostridia and preparation of their neurotoxins. In Montecucco, C (ed.): *Clostridial Neurotoxins*. Berlin: Springer-Verlag, pp 143–160.

Sollner T, Whiteheart SW, Brunner M, Erjument-Bromage H, Geromanos S, Tempst P and Rothman, JE (1993) SNAP receptors implicated in vesicle targeting and fusion *Nature* **362**, 318–324.

Wictome M, Rossetto O, Montecucco C and Shone CC (1996) Substrate residues N-terminal to the cleavage site of BoNT/B play a role in determining the specificity of its endopeptidase activity *FEBS Lett* **386**, 133–136.

Allosteric-type control of synaptobrevin cleavage by tetanus toxin L chain

Fabrice Cornille, Loïc Martin, Christine Lenoir, Bernard P. Roques* and Marie-Claude Fournie-Zaluski

Département de Pharmacochimie Moléculaire et Structurale, U266 INSERM - URA D1500 CNRS, Université René Descartes, UFR des Sciences Pharmaceutiques et Biologiques, 4 Avenue de l'Observatoire, 75270 PARIS Cedex 06, France.

Keywords: Tetanus toxin, clostridial neurotoxins, synaptobrevin, VAMP, botulinum neurotoxins, metallopeptidase, allostery.

The light chain of tetanus neurotoxin (TeNT-L chain) has been shown to be endowed with a zinc endopeptidase activity, selectively directed towards the Gln^{76}-Phe^{77} bond of synaptobrevin, a vesicle-associated membrane protein (VAMP) critically involved in neuroexocytosis. In a previous report, the 50-residue synaptobrevin fragment 39-88 (S 39-88) was shown to be the minimum substrate for the TeNT-L chain, suggesting a role in the mechanism of substrate hydrolysis for residues distal from the cleavage site. In this study, synthetic peptides corresponding to sequences 27-55 (S_1) and 82-93 (S_2) of synaptobrevin were shown to induce a large dose-dependent increase (S_1 : ~170% and S_2 : ~600% at 100 mM) in the cleavage rate of the fluorescent substrate [pyrenylalanine^{88}] S 39-88 by TeNT-L chain. Furthermore, synthetic synaptobrevin fragments S 50-93 and S 32-81 lacking either S_1 or S_2 sequences and not hydrolyzed by the TeNT-L chain were cleaved at a rate comparable to that of the optimal substrate, S 32-93, after addition of 1 mM S_1 or S_2. These effects appeared to result from an activation process mediated by the binding of S_1 and S_2 with complementary sites present on TeNT. All the data can be interpreted by a cooperative exosite-controlled hydrolysis of synaptobrevin by TeNT through an allosteric or induced-fit mechanism. This could account for the high degree of substrate specificity of TeNT and botulinum neurotoxins.

Tetanus toxin (TeNT) and the seven serotypes of botulinum neurotoxins (BoNT/A to /G) are produced by several anaerobic bacilli of the genus *Clostridium* and cause the paralytic syndromes of tetanus and botulism by blocking neurotransmitter release at central and peripheral synapses respectively (Niemann, 1991; Wellhöner, 1992). They are formed by two disulfide-linked polypeptides : the heavy chain H (100 kDa) is responsible for specific binding to neurons and cell penetration while the light chain L (50 kDa) blocks neuroexocytosis after reduction of the disulfide bond (Niemann, 1991; Wellhöner, 1992). The L chain of these neurotoxins has been shown to be endowed with a zinc endopeptidase activity associated with a very high substrate selectivity (Rossetto *et al.*, 1995), contrasting with the wide specificity of zinc proteases belonging to the thermolysin and metzincin families. Indeed, clostridial neurotoxins appear to cleave only a single protein target at a single peptide bond (Rossetto *et al.*, 1995). In the case of TeNT, this target is synaptobrevin, also known as VAMP (for Vesicle-Associated Membrane Protein), a 116-residue integral membrane protein of small synaptic vesicles, highly conserved in eucaryotes (Trimble *et al.*, 1988; Baumert *et al.*, 1989; Südhof *et al.*, 1989). TeNT-L chain cleaves selectively synaptobrevin at the Gln^{76}-Phe^{77} peptide bond (Fig.1) (Schiavo *et al.*, 1992). Successive truncations of synaptobrevin at its N- and C-terminus showed that the synaptobrevin fragment S 39-88 is the minimum efficient substrate, since the removal of 6 residues from the N- or the C-terminus resulted in a dramatic decrease (100 times) in the rate

*To whom correspondence should be sent. Professor Bernard P. Roques, Département de Pharmacochimie Moléculaire et Structurale, U266 INSERM - URA D1500 CNRS, Université René Descartes - UFR des Sciences Pharmaceutiques et Biologiques, 4 Avenue de l'Observatoire - 75270 PARIS Cedex 06, France.
Tel. (33)-1-43.25.50.45. - Fax (33)-1-43.26.69.18.

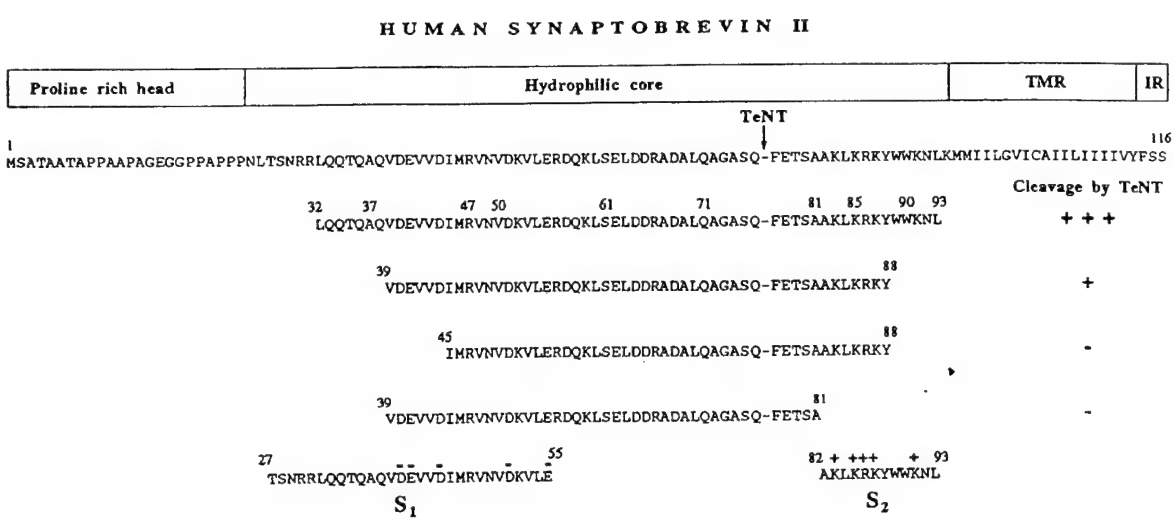


Figure 1. Primary sequence of fragments of human synaptobrevin II used as substrate or modulators of TeNT enzymatic activity. The S prefix is an abbreviation for human synaptobrevin II, followed by the first and the last residue number in the original sequence. Peptides S 27-55 and S 82-93 are designated S_1 and S_2 respectively. The cleavage site Q^{76}-F^{77} of TeNT is indicated by the arrow. TMR indicates the transmembrane region and IR, the intravesicular region. The differences in the cleavage rate of the different substrates are indicated by the presence or absence of the sign (+), and suggested that sequences distal from the cleaved bond Q^{76}-F^{77} are involved in the enzyme substrate interaction.

of cleavage by TeNT-L chain (Fig. 1) (Soleilhac *et al.*, 1996). These data suggested that domains of synaptobrevin, distal from the cleavage site, such as fragments 39-45 (or 25-45) and 82-88 (or 82-93) could play an important role in the hydrolysis of this peptide. In order to understand this role, the corresponding synaptobrevin (S) domain S_1 (for S 27-55) and S_2 (for S 82-93) (Fig. 1) have been synthesized in order to study their influence on the synaptobrevin cleavage by TeNT L chain.

Materials and Methods

Fluorescent measurements of TeNT-mediated cleavage of [Pya^{88}] S 39-88 in presence of various peptides

The enzymatic assay has been described in detail in Soleilhac *et al.* (1996). Briefly, 20 µM of substrate [Pya^{88}] S 39-88 (50 amino acids) and different concentrations of modulatory peptides were incubated in siliconized eppendorfs in 100 µl of 20 mM HEPES (pH 7.4) containing 250 ng of TeNT L chain (50 nM) for 20 min at 37C in the dark. The reaction was stopped by the addition of 900 µl of 72% MeOH 0.1% TFA in H_2O and was then applied to a Sep-Pak Vac C_{18} cartridge (Waters) in order to separate the fluorescent metabolite [Pya^{88}] S 77-88 from the substrate. This metabolite was eluted with 3 ml of 65% MeOH 0.1% TFA in H_2O. The eluate was collected and their fluorescence read at 377 nm after excitation of the fluorophore at 343 nm using a Perkin Elmer LS-50B fluorometer. The amount of released metabolite was calculated from the fluorescence of known concentrations of synthetic metabolite [Pya^{88}] S 77-88. The same assay was used for dose-dependent experiments with S_1 and S_2 peptides and to determine the kinetic constants for enzymatic cleavage in the presence of 100 µM of S_1 and S_2 peptides.

RP-HPLC analyses of TeNT-mediated endoproteolysis of synaptobrevin peptides

For substrate peptides which did not bear the fluorescent probe (S 32-93, S 32-81 and S 50-93), the enzymatic assays were carried out in a typical volume of 100 µl in siliconized eppendorf tubes. In this case, 100 µM of substrate were incubated with 1 µg TeNT-L chain in the presence of 1mM S_1 or S_2 in 20 mM HEPES (pH 7.4) for 20 min at 37C. The cleavage was stopped by adding 50 µl of HCl 0.2M and this solution was analyzed by HPLC on a Capcell C_8 column (300 Å, 7 µm, 250 x 4.6 mm) under isocratic conditions (24% B for the metabolite S 77-93 and 8% B for S 77-81. Eluent A : TFA 0.05% in H_2O ; Eluent B : CH_3CN 90% TFA 0.038% in H_2O, ultraviolet detection k = 214 nm). The amount of metabolite released was determined by HPLC from a standard curve obtained by injection of known

concentrations of synthetic metabolites (S 77-93 and S 77-81).

Results

Dose-dependent activation of the TeNT-L chain-mediated endoproteolysis of [Pya^{88}] S 39-88 by S_1 and S_2

The influence of peptides S_1, S_2 and related synaptobrevin fragments on the enzymatic activity of TeNT was studied by using the fluorescent substrate [Pya^{88}] S 39-88, which contains the non-natural fluorescent amino-acid Pyrenylalanine (Pya) in position 88 (Soleilhac *et al.*, 1996). Increasing concentrations of peptides S_1 and S_2 induced an activation of [Pya^{88}] S 39-88 cleavage, with maximal substrate degradation obtained at a concentration of either S_1 or S_2 of 100 µM (170% and 600% respectively) (Fig. 2). In the same conditions, 100 µM of the acidic synaptobrevin peptides S 37-47, S 61-71 or a basic non-relevant peptide (MQRGNFRNQRKNV) from the nucleocapsid protein of HIV-1 (NCp7 1-13), taken as control, had no effect (data not shown). In all these studies, control experiments by RP-HPLC indicated that the enhanced fluorescence corresponded to an increase in the formation of the metabolite [Pya^{88}] S 77-88 (data not shown). No other cleavage was observed. Both peptides were then used at 100 µM in the same assay in order to measure their influence on the kinetic constants (Km and Vmax) of the enzymatic reaction (Table 1). The addition of peptide S_2 produced a shift of the apparent Michaelis constant (Km) value of the substrate from 576 µM to 51 µM, while the maximal velocity (Vmax) was only slightly affected (166 instead of 111 pmoles/min/g). The potentiating effect induced by the peptide S_1 was lower, the Km shifting from 576 µM to 479 µM and the Vmax shifting from 111 to 169 pmoles/min/g).

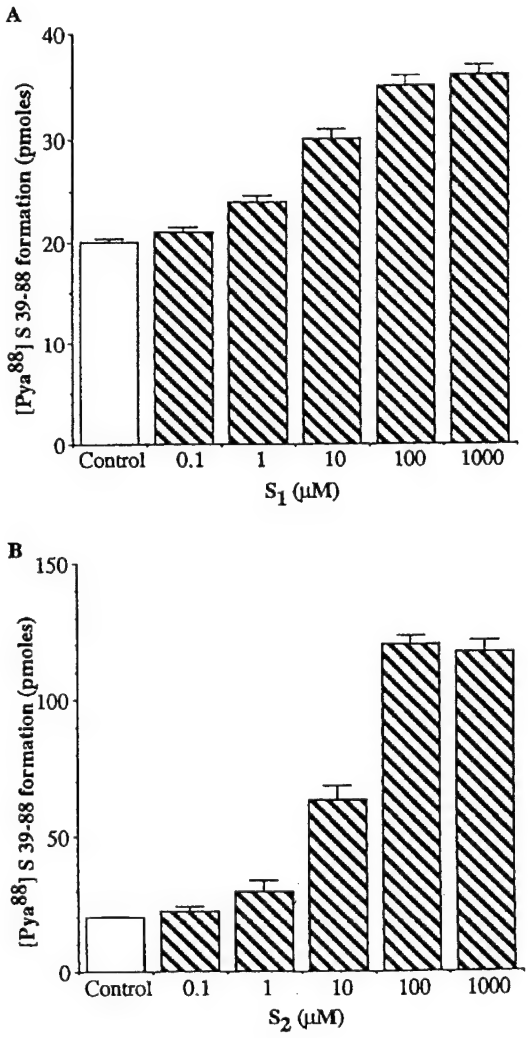


Figure 2. Dose-dependent activation of TeNT-L chain endopeptidase activity by synaptobrevin fragments S_1 and S_2 distal from the cleavage site. 20 µM of [Pya^{88}] S 39-88 were incubated in presence of different concentrations of S_1 (A) and S_2 (B) in 100 µl HEPES 20 mM (pH 7.4) with 250 ng of TeNT-L chain during 20 min at 37C in darkness. The released metabolite [Pya^{88}] S 77-88 was separated from the substrate and quantified by fluorescence spectrometry. Control represents the absence of modulatory peptide.

Table 1. Kinetic constants determination of [Pya^{88}] S 39-88 cleavage by TeNT L chain : influence of either S_1 or S_2.

	KM(uM)	Vmax (pmoles/min/ug)
[Pya^{88}] S 39-88	576 ± 11	111 ± 10
[Pya^{88}] S 39-88 + S_1	479 ± 12	169 ± 20
[Pya^{88}] S 39-88 + S_2	51 ± 11	166 ± 20

100 µM of each peptides S_1 or S_2 were used in these experiments. Each measurement represents the average of three experiments performed in duplicate.

Promoting effects of peptides S_1 and S_2 on the cleavage of fragments S 50-93 and S 32-81 by TeNT-L chain

In order to see if S_1 or S_2 could promote the metabolism of synaptobrevin fragments S 50-93 and S 32-81, which are poorly or not at all cleaved by TeNT-L chain, the latter peptides were incubated in the presence of 1 mM of S_1 or S_2 before addition of TeNT-L chain. The influence of the same concentration of S_1 or S_2 on the cleavage rate of the optimal substrate S 32-93 was evaluated for comparison. The reaction mixtures were analyzed by RP-HPLC in order to quantify the level of metabolites generated. The results are reported in Table 2. The cleavage rate

Table 2. *Influence of S_1 and S_2 on the cleavage rate of peptides S 32-93, S 50-93 and S 32-81 by TeNT-L chain.*

Peptides	Cleavage rate (pmoles/min/ug)		
	Control	+ S_1 (10^{-3}M)	+ S_2 (10^{-3}M)
S 32-93	102 ± 2	105 ± 5	100 ± 3
S 50-93	1.5 ± 0.5	52 ± 5	1.5 ± 0.5
S 32-81	< 0.1	< 0.1	15 2

100 µM of S 50-93 or S 32-93 were incubated in presence or in absence of 1mM of S1 in 100 µl HEPES 20 mM (pH 7.4) with 1 µg of TeNT-L chain (200 nM) during 20 min at 37C.The reaction was stopped by addition of 50 µl of HCl 0.2 N. The released metabolites were quantified by RP-HPLC in isocratic conditions as described in the methods. The values represent the average of three independant experiments performed in duplicate.

of S 50-93 in the presence of 1 mM S_1 increased 34 times (52 pmoles/min/g) reaching a level comparable to that of S 32-93 (102 pmoles/min/g) but was not affected by the addition of 1mM S_2 (Table 2). An even greater potentiating effect was observed following addition of 1 mM S_2 to S 32-81. This effect is difficult to quantify because, in the absence of S_2, the cleavage of S 32-81 was undetectable, but taking 0.1 pmol/min/g as the limit of metabolite detection, it can be assumed to be greater than 150 times (Table 2). The addition of 1 mM S_1 to this peptide did not improve its cleavage. In presence of 1 mM S_1 or S_2, the cleavage rate of the optimal substrate S 32-93 (containing both sequences S_1 and S_2) was not affected (Table 2).

Discussion

The proteolytic activity of tetanus toxin towards synaptobrevin, a protein involved in exocytosis of neurotransmitters, has some unusual properties as compared to that of the classical zinc endopeptidases. The toxin has a very high *in vitro* and *in vivo* selectivity for synaptobrevin, cleaving this peptide exclusively at the Gln^{76}-Phe^{77} bond (Schiavo *et al.*, 1992). Furthermore, the minimal fragment of synaptobrevin, recognized and cleaved by the toxin, corresponds to a large domain of 50 amino-acids (S 39-88) which cannot be reduced without a complete loss of activity (Foran *et al.*, 1994; Cornille *et al.*, 1994; Yamasaki *et al.*, 1994; Soleilhac *et al.*, 1996). All these data suggested that additional interactions involving synaptobrevin domains, far from the cleavage site in the primary sequence, are required for an optimal recognition of the peptide target by the enzyme. This is reminiscent of the mechanism of action of thrombin, a trypsin-like serine protease, which makes use of exosites for recognition of its substrate (Stubbs and Bode, 1995). To explore this hypothesis, various peptides derived from synaptobrevin have been synthesized and their abilities to modify the cleavage rate of the potent synthetic substrate [Pya^{88}] S 39-88 have been studied. The acidic domain S_1 and the basic domain S2 was found to improve the rate of [Pya88] S 39-88 cleavage by a factor 1.7 and 6 respectively. It should be noticed that the shorter acidic peptides S 37-47 (AQVDEVVDIMR) and S 61-71 (SELDDRADALQ) which have been reported to reduce synaptobrevin cleavage by TeNT (Rossetto *et al.*, 1994) have no effects in these conditions. Furthermore, the role played by the two domains S_1 and S_2 in the cleavage of synaptobrevin is underlined by the experiments showing that fragments such as S 50-93 and S 32-81, which are insensitive to TeNT-L chain activity, are hydrolized by addition of the missing S_1 and S_2 sequences respectively. The cleavage rate measured for the mixtures of peptides was lower than that measured with the substrate S 32-93 (Table 2). This is not unexpected, since the affinity of the isolated fragments for their corresponding exosites cannot be as high as when they are included in the substrate sequence.

All these findings support the assumption that peptides S_1 and S_2 favour the cleavage of synaptobrevin fragments by interaction with corresponding exosites present at the toxin surface. However, it was important to exclude a possible promotion by S1 or S2 fragments of a conformational change of an uncleaved peptide, resulting in a structure fitting the TeNT catalytic site with subsequent formation of the Michaelis complex. This explanation is not supported by the fluorescence spectra of S 50-93 and S_2 following addition of the S_1 and S 32-81 peptides respectively, which were not modified and did not reproduce the spectrum of the cleavable sequence S 32-93 (data not shown).

Thus, the present results could be interpreted by an exosite-controlled cleavage of synaptobrevin by TeNT-L chain metallopeptidase activity. The increased apparent affinity of the substrate [Pya^{88}] S 39-88, reflected by a 10 times reduction in Km value in presence of S_2 while the maximal velocity is not significantly affected and the induction of S 32-81 hydrolysis by S_2 are in rather good agreement with an allosteric model of enzyme functioning (Monod *et al.*, 1965; Koshland *et al.*, 1966). Nevertheless, further physico-chemical experiments will be necessary to confirm this assumption. According to this hypothesis, the predominant conformational state of TeNT-L chain in solution could correspond to inactive form(s) in equilibrium with a very low percentage of active form. The population of the active form would be increased thanks to its higher affinity for the S_1 and S_2 domains of synaptobrevin towards

this form, their interaction with the corresponding exosites on TeNT triggering the proteolytic cleavage of this vesicle-associated membrane protein. Such a cooperative mechanism of action is quite uncommon for proteases, with the exception of the serine protease thrombin (Vu *et al.*, 1991), and as far as we know, it has never been described in the case of zinc endopeptidases. This mechanism could be the key of TeNT-L chain and probably of all clostridial neurotoxins specificity.

References

Baumert M, Maycox PR, Navone F, DeCamilli P and Jahn R (1989) Synaptobrevin: an integral membrane protein of 18000 daltons present in small synaptic vesicles of rat brain. *EMBO J* **8**, 379–384.

Cornille F, Goudreau N, Ficheux D, Niemann H and Roques B P (1994) Solid-phase synthesis, conformational analysis and in vitro cleavage of synthetic human synaptobrevin II 1-93 by tetanus toxin L-chain *Eur J Biochem* **222**, 173–181.

Foran P, Shone CC and Dolly JO (1994) Differences in the protease activities of tetanus and botulinum B toxins revealed by the cleavage of vesicle-associated membrane protein and various sized fragments. *Biochemistry* **33**, 15365–15374.

Koshland DE Jr., Némethy G and Filmer D (1966) Comparison of experimental binding data and theorical models in protein containing subunits. *Biochemistry* **5**, 365–385.

Monod J, Wyman J and Changeux JP (1965) On the nature of allosteric transition: a plausible model. *J Mol Biol* **12**, 88, 118.

Niemann H (1991) Molecular biology of clostridial neurotoxins In *A Sourcebook of Bacterial Protein Toxins* (Alouf JE and Freer JH, eds), 303–348, Academic Press, London.

Rossetto O, Deloye F, Poulain B, Pellizari R, Schiavo G and Montecucco C, 1995) The metallo-proteinase activity of tetanus and botulinum neurotoxins. *J.* Physiol (Paris) **89**, 43–50.

Rossetto O, Schiavo G, Montecucco C, Poulain B, Deloye F, Lozzi L. and Shone, C. (1994) SNARE motif and neurotoxins. *Nature* **372**, 415–416.

Schiavo G, Benfenati F, Poulain B, Rossetto O, Polverino de laureto P, Dasgupta B and Monteccuco C (1992) Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin *Nature* **359**, 832–834.

Soleilhac JM, Cornille F, Martin L, Lenoir C, Fournié-Zaluski MC and Roques BP (1996) A sensitive and rapid fluorecence-based assay for determination of tetanus toxin peptidase activity *Anal Biochem* **241**, 120-127.

Stubbs MT and Bode W (1995) The clot thickens: clues provided by thrombin structure *Trends Biochem Sci.* **20**, 23–28.

Südhof TC, Baumert M, Perin MS, and Jahn R (1989) A synaptic vesicle membrane protein is conserved from mammals to drosophila *Neuron* **2**, 1475–1481.

Trimble WS, Cowan DM and Scheller R H (1988) VAMP-1: a synaptic vesicle-associated integral membrane protein *Proc Natl Acad Sci* USA **85**, 4538–4542.

Vu T-KH, Wheaton VI, Hung DT, Charo T and Coughlin SR (1991) Domains specifying thrombin receptor interaction *Nature* **353**, 674–677.

Wellhöner, HH (1992)Tetanus and botulinum neurotoxins. In Herkin H, Hucho F, *Handbook Exp Pharmacol* **102**, 357–417. Berlin, Springer.

Yamasaki S, Baumeister A, Binz T, Blasi J, Link E, Cornille F, Roques BP, Fykse EM, Südhof TC Jahn R and Niemann H (1994) Cleavage of members of the synaptobrevin/VAMP family by types D and F botulinal neurotoxins and tetanus toxin. *J Biol* Chem **269**, 12764–12772.

Session 4

Structure, Action and as Probes II

Multiple alterations of neurotransmission induced by Rab3 and clostridial neurotoxins reveal a functional link between Rab3 and SNAREs.

Frédéric Doussau[1], Ludger Johannes[2,*], Jean-Pierre Henry[2], Bernard Poulain[1] and François Darchen[2]

[1] Laboratoire de Neurobiologie Cellulaire, CNRS, Centre de Neurochimie, 5 rue Blaise Pascal, F-67084 Strasbourg Cedex, France and [2] Service de Neurobiologie Physico-Chimique, CNRS UPR 9071, Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, F-75005 Paris, France

Key words: neuroexocytosis, botulinum neurotoxin type A, tetanus toxin, synaptic proteins, Rab3, small G-protein, SNARE, SNAP-25, VAMP/synaptobrevin; Aplysia

The targets of *Clostridial* neurotoxins are the vesicle protein VAMP/synaptobrevin and two proteins associated with the plasma membrane, SNAP-25 and syntaxin 1. These proteins can associate in a stable ternary complex that forms a receptor for SNAPs (soluble NSF attachment proteins) and are thus referred to as the SNAREs. In exocytosis, they are involved in the docking of vesicles and ensuing fusion steps. Among proteins that might regulate the activity of the SNAREs is Rab3, a small GTP-binding protein implicated in the control of Ca^{2+}-triggered exocytosis. In this study, Rab3 mutant proteins were exploited at neuro-neuronal synapses to investigate the role of Rab3 in neuroexocytosis. Rab3, in its GTP-bound form was found to exert an inhibitory regulation of neuroexocytosis suggesting that GTP hydrolysis by Rab3 is rate-limiting in the exocytosis process. The interference between the action of tetanus (TeNT) or botulinum type A (BoNT/A) neurotoxins and Rab3 mutants were examined. Notably, intracellular pre-injection of a GTPase-deficient Rab3 mutant strongly delayed the inhibition of exocytosis induced by either TeNT or BoNT/A, indicating that the GTPase-deficient Rab3 mutant led to the accumulation of a toxin-insensitive component of release. Since these neurotoxins cannot attack their targets, VAMP/synaptobrevin and SNAP-25, once the latter are engaged into the SNARE complex, these data suggest that the GTP-locked Rab3 mutant induced an accumulation of assembled SNAREs.

Neurotransmitter release results from the exocytosis of the content of synaptic vesicles upon Ca^{2+} influx at nerve terminals. This crucial step for neurotransmission is specifically blocked by the botulinum (BoNTs) and tetanus (TeNT) neurotoxins. This inhibitory action consists in non-proteolytic (Ashton *et al.*, 1995) and proteolytic actions (Schiavo *et al.*, 1992). The three intracellular targets cleaved by TeNT and the various BoNTs are three essential proteins of the fusion apparatus. These are namely i) Vesicle Associated Membrane Protein/synaptobrevin (VAMP/synaptobrevin) a ~18 kDa integral protein of the synaptic vesicle membrane which is specifically attacked by TeNT and BoNT/B, /D, /F or /G, ii) SNAP-25 (synaptosome associated protein of 25 kDa) a synaptic protein associated with the plasma membrane and cleaved by BoNT/A and /E and iii) syntaxin 1, an integral plasma membrane protein of ~35 kDa which is affected only by BoNT/C (reviewed in Niemann *et al.*, 1994; Montecucco and Schiavo, 1995).

VAMP/synaptobrevin, syntaxin 1 and SNAP-25 can bind together in a stable ternary complex (Hayashi *et al.*, 1994) which acts as a receptor for the soluble NSF attachment proteins or SNAPs (Söllner *et al.*, 1993). Hence, the three neurotoxins' targets are termed SNAREs for SNAP-receptor. Due to the hiding of the toxins' recognition sites inside the SNARE complex (Rossetto *et al.*, 1994), VAMP/synaptobrevin, syntaxin 1 and SNAP-25 become

* Present address : Institut Curie, CNRS UMR 144, 12 rue Lhomond, F-75005 Paris, France

Correspondence should be sent to Dr. Bernard Poulain at above address. Tel: (33) 88 45 66 77, Fax: (33) 88 60 16 64, email: poulain@neurochem.u-strabg.fr

resistant to toxin attack once they are complexed (Pellegrini *et al.*, 1994; Hayashi *et al.*, 1994). The pairing of the vesicle and plasma membrane SNAREs is believed to play a key role in the docking of synaptic vesicles at the active zone. Together with SNAPs and NSF = N-ethylmaleimide sensitive factor (N-ethylmaleimide fusion protein), the SNARE complex is likely to be also essential for several ensuing fusion steps (for reviews see Ferro-Novick and Jahn, 1994; Südhof, 1995). However, several regulatory factors should act together with the SNAREs in docking and fusion. Among possible factors are the proteins of the Rab family, which have been implicated in the control of intracellular vesicle traffic, and especially members of the Rab3 subfamily since these monomeric GTP-binding proteins are mainly expressed in neurones and neuroendocrine cells (Darchen *et al.*, 1990; 1995; Fischer von Mollard *et al.*, 1991). As with all members of the Rab family, Rab3 proteins undergo a multi-step cycle (Fig. 1) between a soluble cytosolic fraction and a secretory vesicle-associated state.

Rab3A has been proposed to negatively regulate exocytosis (Holz *et al.*, 1994; Johannes *et al.*, 1994, Henry *et al.*, 1996). However, the molecular basis of this regulatory action and the identity of the step of

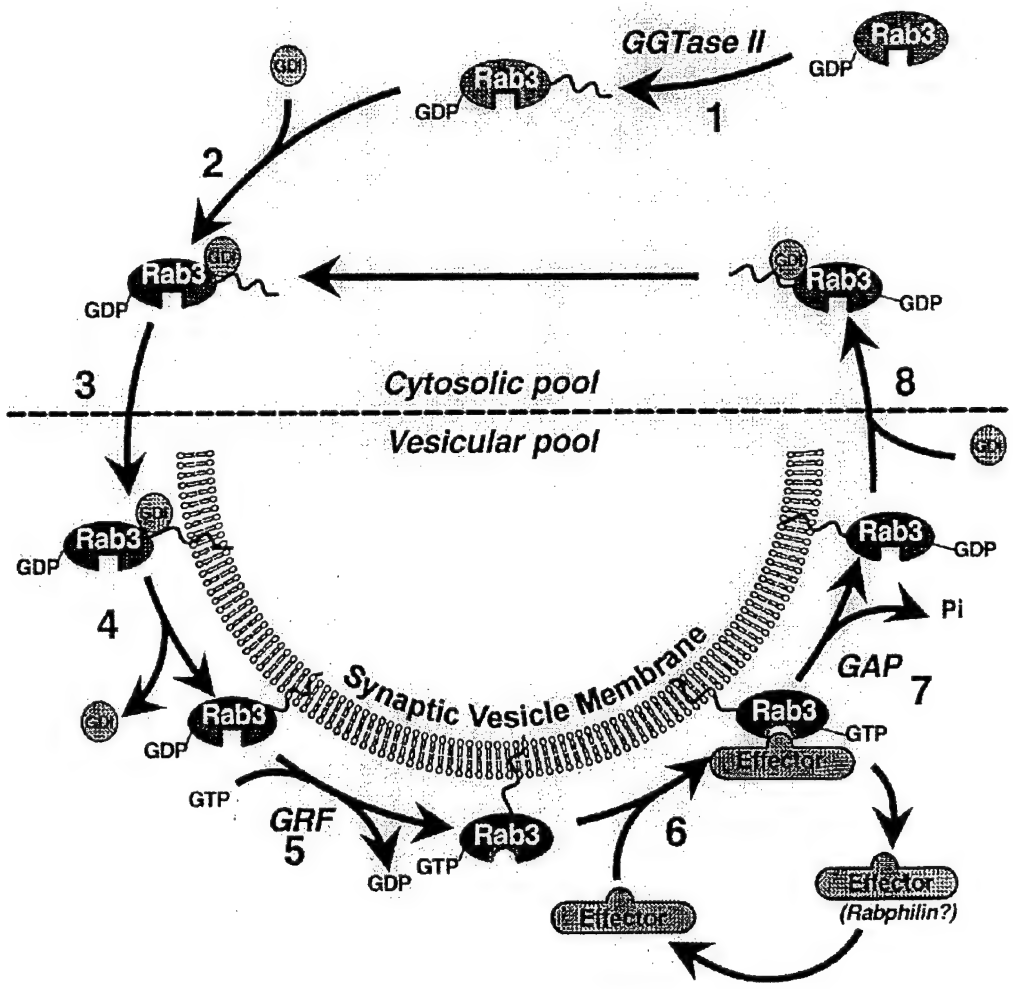


Figure 1 Rab3 cycle. The small GTP binding proteins Rab3 are either found in the cytosol where they are bound to GDP and complexed to Rab-guanine nucleotide dissociation inhibitor (GDI) (step 2), or in association with vesicle membrane (steps 4-7) (mainly under the GTP bound form). By analogy with other Rab proteins, interaction of Rab3 with the synaptic vesicle membrane is followed by release of GDI into the cytosol and by a guanine nucleotide exchange catalysed by GRF (guanine nucleotide releasing factor) (steps 4 and 5). Then, GTP-bound Rab3 interacts with an effector molecule (probably Rabphilin) to control synaptic vesicle targeting to plasma membrane, docking or fusion (step 6). Following GTP hydrolysis catalysed by a GTPase-activating protein (GAP) (step 7), GDP-bound Rab3 is extracted from membrane by Rab-GDI (step 8). Thus, it can enter a new functional cycle.

neuroexocytosis controlled by Rab3 remain to be determined. In the yeast *Saccharomyces cerevisiae* several lines of genetic evidence support a role for Rab proteins in the assembly of the SNARE complex (Søgaard *et al.*, 1994; Lian *et al.*, 1994). Thus, in neurones, it is plausible that Rab3 and the SNAREs are functionally linked. This hypothesis was tested at identified *Aplysia* neuro-neuronal synapses. After having characterised a constitutively active mutant of Rab3, the possible interference between this mutant and treatments which affect selectively the function of the SNAREs were examined.

Materials and Methods

Electrical recordings and neuronal application of Rab proteins or neurotoxins

Experiments were performed at 22°C at identified cholinergic synapses in the buccal ganglion of the marine sea slug *Aplysia californica* (Marinus Inc., CA, USA) using a electrophysiological approach described elsewhere (Schiavo *et al.*, 1992; Ashton *et al.*, 1995, Poulain *et al.*, 1996). Prior to intracellular administration, Rab3 mutants or TeNT samples were mixed with a dye (10% v/v; fast green FCF, Sigma Chem. Co.) and air-pressure injected under visual and electrophysiological monitoring. For extracellular application of BoNT/A, the superfusion was stopped and the toxin added to the bath. After 20 min, superfusion was resumed and all unbound toxin washed away.

Recombinant Rab3 proteins

In contrast to vertebrates where 4 isoforms of Rab3 are known, only one Rab3 gene has been already identified in *Aplysia californica* GenBank database accession number U00986). Comparison of human Rab3A and *Aplysia* Rab3 sequences showed that they diverge in their C-terminal domain, a region thought to be essential for targeting Rab3 to the synaptic vesicle membrane. To take advantage of previous constructs made with human Rab3A (Johannes *et al.*, 1994) and to avoid a mistargeting of recombinant Rab3A in *Aplysia* neurones, the last 24 amino-acids of human Rab3A were replaced by the corresponding residues of *Aplysia californica* Rab3 using a PCR-based strategy (Johannes *et al.*, 1994). This chimera was referred to as hu/apRab3.

In order to disrupt the Rab3 cycle at different steps, punctual mutations were introduced in hu/apRab3. Earlier studies showed that mutational exchange of Gln by Leu at position 81 of Rab3A amino acid sequence (Q81L mutant) leads to a defect in Rab3A GTPase activity (disruption of step 7 in Fig 1). Thus, the Q81L mutation was introduced into hu/apRab3 sequence (hu/apRab3Q81L). To alter the interaction of Rab3 with its effector (disruption of step 6 in Fig 1), the V55E mutation which affects the effector loop of Rab3 was introduced in either hu/apRab3 or hu/apRab3Q81L. The respective recombinant proteins were expressed in *E. coli* as glutathione S-transferase (GST) fusion proteins and purified on glutathione-Sepharose.

Results and Discussion

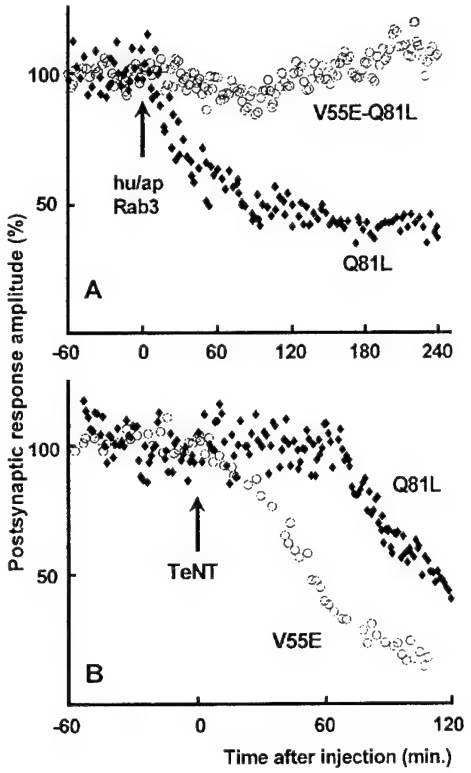


Figure 2 Effect of hu/apRab3 mutants and tetanus toxin on ACh release from *Aplysia* neurones. ACh release was evoked at identified synapses in the buccal ganglion of *Aplysia* and monitored before and after microinjection (at time zero) of different Rab3 mutants into a presynaptic neurone. **A)** Effects of the GTPase deficient (i.e. GTP-locked) Rab3 mutant hu/apRab3Q81L (◆) and abolition of its effect by the mutation V55E (○) in Rab3 effector domain. **B)** Interference between Rab3 mutants and TeNT action. Presynaptic neurones were sequentially injected with either hu/apRab3Q81L (◆) or hu/apRab3V55E (○) (both at 0.4 M final concentration). Then, after stabilisation of the neurotransmitter release (within ~2 hours), TeNT (about 8 nM, final) was subsequently injected into the same neurones. This induced an inhibition of neurotransmission, however, note the long delay in TeNT action after preinjection of Q81L mutant (◆).

GTP hydrolysis by Rab3 is rate-limiting for acetylcholine release at Aplysia synapses

Intraneuronal injection of the GTPase-deficient Rab3 mutant hu/apRab3Q81L (Fig. 2A) into a cholinergic presynaptic neurone at the buccal ganglion of *Aplysia* produced a marked inhibition of acetylcholine (ACh) release. Similar results were obtained after separation of GST and Rab3 moieties by thrombin (not shown). This result is consistent with similar findings obtained in chromaffin cells (Johannes *et al.*, 1994; Holz *et al.*, 1994). The Q81L mutation is known to strongly reduce the intrinsic GTPase activity of Rab3A (Brondyk *et al.*, 1993), and Rab3Q81L thus remains 'locked' under the GTP-bound form. Hence, our results suggest that GTP hydrolysis by Rab3 is rate-limiting during neuroexocytosis.

The effect of Rab3Q81L is supposed to be mediated by the intraneuronal Rab3 effector. Therefore, the Q81L effect should be suppressed by altering the Rab3 domain (i.e. the effector loop) implicated in Rab3/effector interaction (step 6 of Fig. 1). The V55E mutation was introduced in hu/apRab3Q81L and indeed, intraneuronal application of the double mutant hu/apRab3(V55E-Q81L) did not lead to any detectable inhibition of ACh release (Fig. 2A). Moreover, as it was seen with the single mutant hu/apRab3V55E, a slight but reproducible potentiation of release was detected ($+21\%$; $n=6$). This potentiation suggests that the V55E mutants enter Rab3 cycle, compete with endogenous Rab3 and prevent the activity of endogenous GTP-bound Rab3 to occur. These data are in a good agreement with the enhancement of the secretory activity observed earlier in chromaffin cells loaded with antisense oligodeoxynucleotides directed to Rab3A mRNA (Johannes *et al.*, 1994).

Functional linkage between Rab3 and the SNAREs

In order to gain evidence on a possible functional linkage between Rab3 and the SNAREs, we examined the interference between the effect of the GTP-locked Rab3 mutant (hu/apRab3Q81L) and of clostridial toxins on ACh release. After stabilisation of inhibition of ACh release by hu/apRab3Q81L, TeNT was injected intraneuronally. Strikingly, ACh release remained unchanged for a $\sim 80 \pm 20$ minutes ($n = 4$); then the inhibition developed (Fig. 2B). This is in contrast with the short delay that follows injection of TeNT alone (14 ± 3 minutes, $n=6$, not shown). Importantly, this action appeared specific for the active form of Rab3 (hu/apRab3Q81L) because preinjection of the Rab3 mutant defective in its effector loop (V55E) had no effect on TeNT-induced inhibition of ACh release (Fig. 2B).

The delayed action of TeNT after preinjection of the GTPase-deficient Rab3 mutant indicates that this mutant reduced the accessibility of the toxin to VAMP/synaptobrevin. Because VAMP/synaptobrevin becomes uncleavable by TeNT once it is engaged within the SNARE complex (Pellegrini *et al.*, 1994; Hayashi *et al.*, 1994), our results suggest that hu/apRab3Q81L induces an accumulation of the SNARE complex itself. To exclude the possibility that VAMP/synaptobrevin was protected against TeNT by an interaction with other synaptic proteins, the ability of hu/apRab3Q81L to protect neurotransmission against BoNT/A (that cleaves SNAP-25) was examined. Again, pre-injection of hu/apRab3Q81L delayed BoNT/A action (by 58 minutes ± 21, $n = 4$; not shown). Only the SNARE complex contains simultaneously VAMP/synaptobrevin and SNAP-25. Hence, our results strongly suggest that GTP-locked Rab3 protects the SNAREs from toxin-induced cleavage by favouring their associated state.

To date, no physical interaction between Rab proteins and the SNAREs has been demonstrated. However, genetic and biochemical evidence in yeast suggest that Rab3 analogues in Ypt/Sec4 family are implicated in the formation or stabilisation of the SNARE complex (Søgaard *et al.*, 1994; Lian *et al.*, 1994). The proposed accumulation of assembled SNAREs (as detected by the temporary insensitivity to the toxins) induced by hu/apRab3Q81L might result from an increased rate of complex assembly. Another possibility, which is consistent with the Rab3Q81L-induced inhibition of release, would be that GTP-locked Rab3 promotes the accumulation of the SNARE-complex by reducing its clearance. According to this hypothesis, GTP hydrolysis by Rab3 would occur downstream of/from the SNARE complex formation, i.e. downstream from/of the docking of synaptic vesicles at the plasma membrane. The reduced GTPase activity of Rab3Q81L would thus account for the observed inhibition of ACh release and for the accumulation of a toxin-insensitive component of release.

Conclusion

Since physical interaction between Rab3 and the SNAREs cannot be demonstrated although a functional linkage can be clearly established (this paper), the existence of an intermediate component between the SNAREs and Rab3 is likely. Rab3 action appears dual: the data obtained in yeast implicate Rab proteins in the SNARE complex formation and our data argue for an additional later action, downstream of the SNARE complex formation (i.e. after

synaptic vesicles are docked). This second Rab3 action would be rate-limiting in exocytosis and determined by the GTPase activity. According to the Rab3 cycle (Fig. 1), Rab3 would leave the vesicle membrane after the docking step.

Acknowledgments

We thank Dr C. Montecucco for his kind gift of TeNT and BoNT/A, L.J. is supported by a fellowship from Boehringer Ingelheim Fonds. This work was supported by research contracts from Association Française de Lutte contre les Myopathies, from Fondation pour la Recherche Médicale (to B.P.), and from Association pour la Recherche sur le Cancer (to J. P. H.).

References

Ashton AC, Li Y, Doussau F, Weller U, Dougan G, Poulain B and Dolly JO (1995) Tetanus toxin inhibits neuroexocytosis even when its Zn^2+-dependent protease activity is removed. *J Biol Chem* **270**, 31386–31390.

Brondyk WH, McKiernan CJ, Burstein ES and Macara IG (1993) Mutants of Rab3A analogous to oncogenic Ras Mutants. *J Biol Chem* **268**, 9410–9415.

Darchen F, Zahraoui A, Hammel F, Monteils M-P, Tavitian A and Scherman D (1990). Association of the GTP-binding protein Rab3A with bovine adrenal chromaffin granules. *Proc Natl Acad Sci USA* **87**, 5692–5696.

Darchen F, Senyshyn J, Brondyk WH, Taatjes DJ, Holz RW, Henry JP, Denizot JP and Macara IG (1995). The GTPase Rab3a is associated with large dense core vesicles in bovine chromaffin cells and rat PC12 cells. *J Cell Sci* **108**, 1639–1649.

Ferro-Novick S and Jahn R (1994) Vesicle fusion from yeast to man. *Nature* **370**, 191–193.

Fischer von Mollard G, Südhof TC and Jahn R (1991) A small GTP-binding protein dissociates from synaptic vesicles during exocytosis. *Nature* **349**, 79–81.

Hayashi T, McMahon H, Yamasaki S, Binz T, Hata Y, Südhof TC and Niemann H (1994) Synaptic vesicle membrane fusion complex: action of clostridial neurotoxins on assembly *EMBO J* **13**, 5051–5061.

Henry JP, Johannes L, Doussau F, Poulain B and Darchen F (1996) Role of Rab3A in neurotransmitter and hormone release : a discussion of recent data. *Biochem Soc Trans* (in press).

Holz RW, Brondyk WH, Senter RA, Kuzion L and Macara IG (1994) Evidence for the involvement of Rab3A in Ca^2+-dependent exocytosis from adrenal chromaffin cells *J Biol Chem* **269**, 10229–10234.

Johannes L, Lledo P-M, Roa M, Vincent J-D, Henry J-P and Darchen F (1994). The GTPase Rab3A negatively controls calcium-dependent exocytosis in neuroendocrine cells *EMBO J* **13**, 2029–2037.

Lian JP, Stone S, Jiang Y, Lyons P and Ferro-Novick S (1994) Yptlp implicated in v-SNARE activation *Nature* **372**, 698–701.

Niemann H, Blasi J and Jahn R (1994) Clostridial neurotoxins: new tools for dissecting exocytosis *Trends Cell Biol* **4**, 179–185.

Montecucco C and Schiavo G (1995) Structure and function of tetanus and botulinum neurotoxins *Quart Rev Biophys* **28**, 423–472.

Pellegrini LL, O'Connor V and Betz H (1994) Fusion complex formation protects synaptobrevin against proteolysis by tetanus toxin light chain *FEBS Lett* **353**, 319–323.

Poulain B, de Paiva A, Deloye F, Doussau F, Tauc L, Weller U and Dolly JO (1996) Differences in the multi-step process of inhibition of neurotransmitter release induced by tetanus toxin and botulinum neurotoxins type A and B at *Aplysia* synapses *Neuroscience* **70**, 567–576.

Rossetto O, Schiavo G, Montecucco C, Poulain B, Deloye F, Lozzi L and Shone C (1994) SNARE motif and neurotoxins *Nature* **372**, 415–416.

Schiavo G, Poulain B, Rossetto O, Benfenati F, Tauc L and Montecucco C (1992) Tetanus toxin is a zinc protein and its inhibition of neurotransmitter release and protease activity depend on zinc *EMBO J* **11**, 3577–3583.

Søgaard M, Tani K, Ye RR, Geromanos S, Tempst P, Kirchhausen T, Rothman JE and Söllner T (1994) A Rab protein is required for the assembly of SNARE complexes in the docking of transport vesicles *Cell* **78**, 937–948.

Söllner T, Whitehart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P and Rothman, JE (1993) SNAP receptors implicated in vesicle targeting and fusion *Nature* **362**, 318–324.

Südhof TC (1995) The synaptic vesicle cycle: a cascade of protein-protein interactions *Nature* **375**, 645–653.

Effects of tetanus toxin light chain on *Drosophila* exocytosis

Cahir J. O'Kane[1], Alexandra Brunner, Brian D. McCabe and Sean T. Sweeney

Department of Genetics, University of Cambridge, Downing Street, Cambridge, CB2 3EH, UK

Keywords: neuromuscular junction; synaptobrevin; cellubrevin; proteolysis; synaptic vesicles

In order to study synaptic vesicle release and to inactivate specific neurons for behavioural studies, we have expressed tetanus toxin light chain (TeTxLC) in transgenic *Drosophila*. Expression of TeTxLC in the CNS causes a complete block of synaptic transmission at the neuromuscular junction, but does not have any other obvious effects on neuronal development or morphology. This is in contrast to mutations which remove either syntaxin or Rop/Munc18, which appear to have more severe defects which also affect exocytosis in general. *In vitro*, TeTxLC cleaves *Drosophila* neuronal synaptobrevin (n-syb) both *in vitro* and *in vivo*, but at least *in vitro* does not cleave the ubiquitously expressed syb protein, which may be functionally similar to cellubrevin. These data are in keeping with a model in which n-syb plays a role which is specific to synaptic vesicle exocytosis, whereas syb plays a similar, though not necessarily identical, role in other situations. We have recently identified mutant alleles of the *syb* gene, which should allow us to test some aspects of this model. Expression in the CNS of two mutant forms of TeTxLC, which carry amino acid substitutions which disrupt protease activity *in vitro*, does not prevent synaptic transmission *in vivo*; individuals carrying these mutant toxin genes are viable. This suggests that at least in *Drosophila*, the protease activity of TeTxLC is both necessary and sufficient to explain its toxicity, although it does not eliminate the possibility of other substrates for the protease activity of TeTxLC. Attempts to rescue TeTxLC toxicity by using mutant synaptobrevins that are resistant to toxin cleavage in vitro have so far failed.

Genetic, biochemical and physiological work in a variety of eukaryotes, including budding and fission yeast, the nematode *Caenorhabditis elegans*, the fruitfly *Drosophila*, and mammals, has identified a plethora of gene products involved in regulating or mediating the process of exocytotic vesicle fusion. Approaches that are largely biochemical in nature have succeeded in defining the molecular properties of many of these gene products, and the processes in which they are involved. However, these are most informative when complemented by genetic approaches. The latter can offer effective ways to manipulate or eliminate expression of specific gene products, thus allowing conclusions about their function in the whole organism, in processes that may be difficult to mimic effectively *in vitro* or in culture. Furthermore, organisms that lack specific gene products can provide an assay system for exogenously added wild-type or manipulated forms of the gene in question, to analyse its role in these processes.

For the study of synaptic exocytosis, *Drosophila* offers both sophisticated genetic tools and a neuromuscular junction easily accessible for electrophysiology. Techniques for recording at central synapses are however less well developed. It has therefore yielded the most informative collection of mutant phenotypes affecting synaptic exocytosis found in any organism to date. Among the genes identified in flies are homologs of the cytosolic vesicle fusion factors SNAP, NSF (2 genes), the SNAREs synaptobrevin (neuronal and ubiquitous forms), syntaxin and SNAP-25, as well as synaptotagmin, the Munc-18/SEC1 homolog *Rop*, rab3 and cysteine string protein (Boulianne and Trimble, 1995; DiAntonio *et al.*, 1993a; DiAntonio *et al.*, 1993b; Harrison *et al.*, 1994; Littleton *et al.*, 1993; Ordway *et al.*, 1994; Pallanck *et al.*, 1995; Risinger *et al.*, 1993; Salzberg *et al.*, 1993; Schulze *et al.*, 1995; Südhof *et al.*, 1989; Zinsmaier *et al.*, 1994).

[1]Corresponding author: Tel: 44-1223-333177; Fax: 44-1223-333177. E-mail c.okane@gen.cam.ac.uk.

The identification of the proteolytic substrates of the various clostridial neurotoxins has shown the important role of the SNARE proteins synaptobrevin, syntaxin and SNAP-25 in synaptic vesicle release. However, there is still remaining uncertainty on a number of issues. Firstly, there are apparently conflicting results on whether tetanus toxin light chain (TeTxLC) may act by stimulating transglutaminase activity in a manner that is independent of its proteolytic activity (e.g. Facchiano *et al.*, 1993; Poulain *et al.*, 1993; Coffield *et al.*, 1994; Ashton *et al.*, 1995). Secondly, while TeTxLC protease shows a high degree of substrate specificity, it is difficult to exclude the possibility of other proteolytic substrates. Thirdly, both flies and vertebrates have two or more genes that encode neuronal and ubiquitous forms of synaptobrevin, and the relative roles of each of these forms have not yet been comprehensively characterised.

Targets of TeTxLC in *Drosophila*, and effects of removing neuronal synaptobrevin

In order to study further the biological effects of TeTxLC, the roles of the two known synaptobrevin homologs in flies, and in addition to inactivate specific neurons for behavioural studies, we investigated the effects of TeTxLC both *in vitro* on its two most likely potential substrates, and *in vivo* in transgenic *Drosophila* (Sweeney *et al.*, 1995).

The two potential most likely substrates for TeTxLC action in *Drosophila* are a neuronal synaptobrevin homolog (*n-syb*), and an ubiquitously expressed homolog (*syb*). These are encoded by two paralogous genes (i.e. homologous genes in the same species; Südhof *et al.*, 1989; DiAntonio *et al.*, 1993a). The neuronal expression of *n-syb* suggested that it might be essentially the fly functional equivalent of the vertebrate synaptobrevin 1 and synaptobrevin 2 isoforms. Work described and reviewed here supports this interpretation. The ubiquitous expression of *syb* suggested that it might be the fly functional equivalent of vertebrate cellubrevin. However, this idea does not receive strong support from sequence-gazing, and will require further experimentation. Evolutionary comparisons of the different synaptobrevin family sequences, (Fig. 1) shows that fly *syb* and *n-syb* are more closely related to each other than either is to any of the vertebrate genes. The relationship of vertebrate cellubrevin to the other family members is not entirely clear. On the face of things, this would suggest that the duplication events that gave rise to neuronal and ubiquitously expressed forms of the synaptobrevins took place independently in the fly and vertebrate lineages, but an earlier timing for the duplication event cannot be excluded.

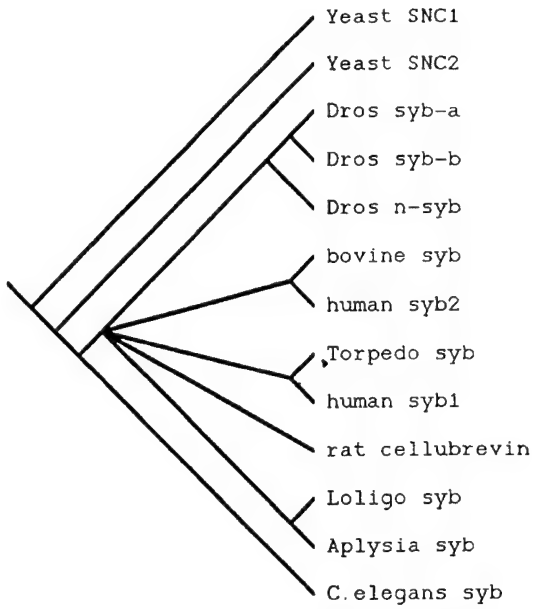


Figure 1. Parsimonous tree showing relatedness of synaptobrevin protein sequences. This tree is a strict consensus of four possible trees generated using heuristic settings of Paup 3.3. and drawn using MacClade 3.0. NCBI Accession numbers are (in order top to bottom) 172655, 311159, 290283, 290282, 436306, 433076, 338632, 135096, 338625, 449148, 437856, 392975 & 549022. *Drosophila syb-a* and *syb-b* are two different splice variants of the *syb* gene (Chin *et al.*, 1993). The origins of the five branches that comprise the *Drosophila*, vertebrate and mollusc sequences cannot be reliably resolved.

The phenomenon of concerted evolution means that paralogous genes in the same species are often more similar than one would expect given their true divergence time. Of course, the possibility of further family members in both vertebrates and flies cannot be excluded.

Fly syb and n-syb proteins were expressed as ^{35}S-labelled products in an *in vitro* transcription-translation expression system. Treatment of these products with either TeTxLC or reduced TeTx led to cleavage of n-syb (albeit less efficiently than cleavage of rat synaptobrevin 2), but no detectable cleavage of syb, even after incubations of up to 6 hours (Sweeney *et al.*, 1995). This is in contrast to the case in vertebrates, where both synaptobrevin and cellubrevin are cleaved by the toxin (Schiavo *et al.*, 1992; Link *et al.*, 1992; McMahon *et al.*, 1993). The resistance of syb protein to TeTxLC cleavage cannot be ascribed to an alteration in the cleavage site used by TeTxLC, as the amino acid sequence GASQFE that includes the QF cleavage site is conserved in both the syb and n-syb proteins, as well as in the susceptible vertebrate proteins. However, a short motif (the SNARE motif) has been found in multiple copies in synaptobrevin,

syntaxin and SNAP 25. This has the consensus HAAXHAXHP (H, hydrophobic; A, acidic; P, polar; X, any amino acid) and has been proposed to form an alpha-helical structure that can be recognised by clostridial neurotoxins (Rossetto *et al.*, 1994). While this model must remain tentative until structural information on SNARE complexes is available, mutations in acidic residues of these SNARE motifs do affect toxin susceptibility selectively (see contributions from Montecucco group these proceedings). Interestingly, fly syb protein carries G residues at positions 60 (in the first of two SNARE motifs in synaptobrevin) and 80 (in the second SNARE motif), rather than the acidic D found in most other homologs at these positions; these changes might therefore account for this protein's resistance to TeTxLC.

These *in vitro* experiments therefore suggested that expression of TeTxLC in flies might specifically inactivate n-syb, but not syb protein. This is in contrast to the case in mammals, where TeTxLC cleaves cellubrevin and can cause morphological defects in non-neuronal cells, possibly as a consequence of this (McMahon *et al.*, 1993; Eisel *et al.*, 1993; Ikonen *et al.*, 1995). We therefore set out to express TeTxLC in both neuronal and non-neuronal cell-types in transgenic flies. To get around the difficulty of generating stable transgenic lines that carried such a potentially deleterious gene, we fused a TeTxLC gene (a synthetic gene obtained from H. Niemann; Eisel *et al.*, 1993) to a promoter containing a UAS (upstream activation sequence) whose activity is dependent on the presence of the yeast transcriptional activator GAL4. Fly lines carrying the UAS-TeTxLC construct did not express toxin at detectable levels because they lacked GAL4 protein. To obtain tissue-specific expression of TeTxLC, the UAS-TeTxLC flies were crossed to a set of other fly lines, each of which expressed GAL4 in a tissue-specific manner (Brand and Perrimon, 1993). The progeny of each of these crosses therefore expressed TeTxLC specifically in those tissues where GAL4 was expressed, and toxin expression could be detected immunohistochemically.

Expression of TeTxLC in the CNS during embryonic development caused a complete block of evoked excitatory junction currents (EJCs) at the neuromuscular junction, but did not have any other obvious effects on neuronal development or morphology. Detectable n-syb immunoreactivity was removed, but the use of other synaptic markers showed normal synaptic morphology (Sweeney *et al.*, 1995). These embryos developed normally, but in late embryogenesis showed paralysis of the body wall muscles and failed to hatch as larvae. This is in contrast to mutations that remove either *syntaxin* or *Rop*, which cause morphological defects consistent with more general defects in exocytosis. Expression of TeTxLC postsynaptically in the muscle had no effect on evoked EJCs, and did not cause paralysis. In fact, these embryos hatched as larvae, survived through all three larval instars and most of pupation, but failed to hatch as adults, presumably due to more widespread CNS expression of TeTxLC in this line in pupal stages.

These results show firstly that none of the thousands of proteins that are necessary for survival and normal development of neuronal and muscle cells in *Drosophila* is inactivated by TeTxLC. Secondly, they support the *in vitro* data that syb is not cleaved by the toxin; in heterozygous $syb^{-}/+$ mutant stocks, a quarter of the embryonic progeny (presumably the syb^{-}/syb^{-} homozygotes) show defects in the arrangement and axonal pathfinding of peripheral neurons, which are not seen in either wild-type or TeTxLC-expressing stocks. Thirdly, they show that the role of n-syb protein is confined to synaptic vesicle release, and that it does not play a role in exocytosis in general, unlike its partner syntaxin. We suggest that syb protein acts as a partner for syntaxin and SNAP-25 in exocytotic events other than synaptic vesicle exocytosis, and that the role of n-syb is specific to synaptic vesicle release, e.g. perhaps in helping to couple the basic vesicle fusion machinery to the Ca^{2+}-sensing machinery.

Proteolytic activity of TeTxLC is required for toxicity in *Drosophila*

Protease-deficient mutant forms of TeTxLC (H237A, E234A, and to a lesser extent H233A) have been shown to retain some ability to inhibit neurotransmitter release in both *Aplysia* neurons and bovine synaptosome preparations, with up to about 50% of the activity of the wild-type toxin in some circumstances (Ashton *et al.*, 1995). We therefore examined the effect of expressing two protease-deficient mutant forms of TeTxLC in *Drosophila*. The mutations used were a double mutation which abolished zinc binding, H233V,H237V, and a single mutation which did not abolish zinc binding, in a residue thought to be required for coordinating a water molecule, E234Q (Yamasaki *et al.*, 1994).

Expression of either the H233V, H237V or the E234Q mutant forms throughout the embryonic CNS had no effect on viability. These embryos hatched as larvae around the normal time (22-24 hours after fertilisation at 25 °C), and developed to adulthood normally in spite of continuing expression of the mutated toxins throughout most of their CNS. These individuals were not noticeably sluggish as either late embryos, larvae, or adults. Experiments to confirm that synaptic transmission appears normal by electrophysiological criteria are now in progress.

The lack of obvious toxicity of protease-deficient TeTxLC mutant forms suggests that at least in *Drosophila*, the protease activity of TeTxLC is both necessary and sufficient for its toxicity. However, while the experiments described in the previous section eliminate thousands of proteins as possible targets, they do not formally eliminate the possibility of further functional proteolytic targets for the toxin. We have therefore attempted to rescue TeTxLC toxicity by expressing an altered synaptobrevin that carries a mutation in the TeTxLC cleavage site (Q76V) and are resistant to toxin cleavage in vitro. To date we have failed to obtain rescue, but we have been unable to detect significant levels of the mutant synaptobrevin in synapses. While a number of explanations are possible, one might be that the Q76V mutant cannot be localised properly in flies. These experiments are therefore inconclusive, but highlight the requirement for an assay for synaptobrevin function.

Conclusions

Our experiments suggest that the effect of TeTxLC in *Drosophila* is limited to abolition of synaptic transmission, and that this effect can be ascribed to proteolytic cleavage of n-syb, but not syb protein. We can find no evidence for a non-proteolytic mechanism of action of the toxin in *Drosophila*. The phenotypes we observe suggest that the role of neuronal synaptobrevin is limited to synaptic vesicle release, and is not involved in other modes of exocytosis.

Acknowledgements

We acknowledge the collaboration of Heiner Niemann, who generously provided us with clones, antibodies, information and advice. Kendal Broadie carried out the electrophysiological analysis of the embryonic NMJ, described in Sweeney *et al.* (1995) and Broadie *et al.* (1995). Isolation of *syb* mutants was performed in collaboration with Yiquan Guo, Kim Kaiser and Eliot Goldstein, and we thank Tom Schwarz for generous exchange of information and materials.

References

Ashton AC, Li Y, Doussau F, Weller U, Dougan G, Poulain B and Dolly JO (1995) Tetanus toxin inhibits neuroexocytosis even when its Zn^{2+}-dependent protease activity is removed *J Biol Chem* **270**, 31386–31390.

Boulianne GL and Trimble WS (1995) Identification of a second homolog of *N*-ethylmaleimide-sensitive fusion protein that is expressed in the nervous system and secretory tissues of *Drosophila Proc Natl Acad Sci USA* **92**, 7095–7099.

Brand AH and Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes *Development* **118**, 401–415.

Broadie K, Prokop A, Bellen HJ, O'Kane CJ, Schulze K and Sweeney ST (1995) Syntaxin and synaptobrevin function downstream of vesicle docking in Drosophila *Neuron* **15**, 663–673.

Chin AC, Burgess RW, Wong BR, Schwarz TL and Scheller RH (1993) Differential expression of transcripts from *syb*, a *Drosophila melanogaster* gene encoding VAMP (synaptobrevin) that is abundant in non-neuronal cells *Gene* 131, 175–181.

Coffield JA, Considine RV, Jeyapaul J, Maksymowych AB, Zhang R and Simpson LL (1994) The role of transglutaminase in the mechanism of action of tetanus toxin *J Biol Chem* **269**, 24454–24458.

DiAntonio A, Burgess RW, Chin AC, Deitcher DL, Scheller RH and Schwarz TL (1993a) Identification and characterization of Drosophila genes for synaptic vesicle proteins *J Neurosci* **13**, 4924–4935.

DiAntonio A, Parfitt KD and Schwarz TL (1993b) Synaptic transmission persists in *synaptotagmin* mutants of Drosophila *Cell* **73**, 1281–1290.

Eisel U, Reynolds K, Riddick M, Zimmer A, Niemann H and Zimmer A (1993) Tetanus toxin light chain expression in Sertoli cells of transgenic mice causes alterations of the actin cytoskeleton and disrupts spermatogenesis *EMBO J* **12**, 3365–3372.

Facchiano F, Valtorta F, Benfenati F and Luini A (1993) The transglutaminase hypothesis for the action of tetanus toxin *TIBS* **18**, 327–329.

Harrison SD, Broadie K, van de Goor J and Rubin GM (1994) Mutations in the Drosophila *Rop* gene suggest a function in general secretion and synaptic transmission *Neuron* **13**, 555–566.

Ikonen E, Tagaya M, Ullrich O, Montecucco C and Simons K (1995) Different requirements for NSF, SNAP, and Rab proteins in apical and basolateral transport in MDCK cells *Cell* **81**, 571–580.

Link E, Edelmann L, Chou JH, Binz T, Yamasaki S, Eisel U, Baumert M, Südhof TC, Niemann H and Jahn R (1992) Tetanus toxin action: inhibition of neurotransmitter release linked to synaptobrevin *Biochem Biophys Res Comm* **189**, 1017–1023.

Littleton JT, Stern M, Schule K, Perin M and Bellen HJ (1993) Mutational analysis of Drosophila *synaptotagmin* demonstrates its essential role in Ca^{2+}-activated neurotransmitter release *Cell* **74**, 1125–1134.

McMahon HT, Ushkaryov YA, Edelmann L, Link E, Binz T, Niemann H, Jahn R and Südhof TC (1993) Cellubrevin is a ubiquitous tetanus-toxin substrate homologous to a putative synaptic vesicle fusion protein *Nature* **364**, 346–349.

Ordway RW, Pallanck L and Ganetzky B (1994) Neurally expressed *Drosophila* genes encoding homologs of the NSF and SNAP secretory proteins *Proc Natl Acad Sci USA* **91**, 5715–5719.

Pallanck L, Ordway RW, Ramaswami M, Chi WY, Krishnan KS and Ganetzky B (1995) Distinct roles for N-ethylmaleimide-sensitive fusion protein (NSF) suggested by the identification of a 2nd *Drosophila* NSF homolog *J Biol Chem* **270**, 18742–18744.

Poulain B, Rossetto O, Deloye F, Schiavo G, Tauc L and Montecucco C (1993) Antibodies against rat brain vesicle-associated membrane protein (synaptobrevin) prevent inhibition of acetylcholine release by tetanus toxin or botulinum neurotoxin type B *J Neurochem* **61**, 1175–1178.

Risinger C, Blomqvist AG, Lundell I, Lambertsson A, Nässel D, Pieribone VA, Brodin L and Larhammar D (1993) Evolutionary conservation of synaptosome-associated protein 25 kDa (SNAP-25) shown by *Drosophila* and *Torpedo* cDNA clones *J Biol Chem* **268**, 24408–24414.

Rossetto O, Schiavo G, Montecucco C, Poulain B, Deloye F, Lozzi L and Shone CC (1994) SNARE motif and neurotoxins *Nature* **372**, 415–416.

Salzberg A, Cohen N, Halachmi N, Kimchie Z and Lev Z (1993) The *Drosophila Ras2* and *Rop* gene pair: a dual homology with a yeast *Ras*-like gene and a suppressor of its loss-of-function phenotype *Development* **117**, 1309–1319.

Schiavo G, Benfenati F, Poulain B, Rossetto O, Polverino de Laureto P, DasGupta BR and Montecucco C (1992) Tetanus and botulinum-B neurotoxins block neurotransmitter release by cleavage of synaptobrevin *Nature* **359**, 832–835.

Schulze K, Broadie K, Perin MS and Bellen HJ (1995) Genetic and electrophysiological studies of Drosophila syntaxin-1A demonstrate its role in nonneuronal secretion and neurotransmission *Cell* **80**, 311–320.

Südhof TC, Baumert M, Perin MS and Jahn R (1989) A synaptic vesicle protein is conserved from mammals to Drosophila *Neuron* **2**, 1475–1481.

Sweeney ST, Broadie K, Keane J, Niemann H and O'Kane CJ (1995) Targeted expression of tetanus toxin light chain in Drosophila specifically eliminates synaptic transmission and causes behavioral defects *Neuron* **14**, 341–351.

Yamasaki S, Hu Y, Binz T, Kalkuhl A, Kurazono H, Tamura T, Jahn R, Kandel E and Niemann H (1994) Synaptobrevin/vesicle-associated membrane protein (VAMP) of *Aplysia californica*: structure and proteolysis by tetanus toxin and botulinal neurotoxins type D and F *Proc Natl Acad Sci USA* **91**, 4688–4692.

Zinsmaier K, Eberle KK, Buchner E, Walter N and Benzer S (1994) Paralysis and early death in cysteine string protein mutants of *Drosophila Science* **263**, 977–980.

Session 5

Therapeutic applications

Botulinum toxin treatment of ocular alignment disorders

Alan B. Scott

Smith Kettlewell Eye Research Institute, 2232 Webster Street, San Francisco, California, 94115-1821 USA

Many useful drugs come from originally toxic sources – digitalis, coumarin and ergot come to mind. Botulinum poisoning has been known since antiquity. It is a surprise that its use to reduce muscle action has only been explored recently. This paper is a brief account of the development of this drug, its uses, effects and complications, and a view of future drug applications using botulinum toxin.

Historical Perspective

The first thorough investigation of botulinum toxin was undertaken by Justinus Kerner and published in 1817-1822 (see Gruesser, 1986). He followed the clinical course of dozens of patients with many important contributions to diagnosis, prognosis and treatment. More importantly, he extracted the toxin (usually from some sausage product), showed its effect in graded doses on various animals, correctly concluded that it paralyzed skeletal and parasympathetic function and proposed its use as a therapeutic agent in neurologic diseases characterized by excessive motor movements, such as chorea. His 'research grant application' (a pleading letter to the King for funds) to research this compound and its potential medical effects and use was presumably turned down, for he rather abruptly abandoned these researches and turned toward medical practice and romantic poetry, a scenario not foreign to us nowadays.

Our research began in 1970/71 with injection of various drugs into extraocular muscle as an alternative to surgical treatment for strabismus. Among these was botulinum toxin, considered earlier by others (Crone, Bach-y-Rita, Jampolsky), but actually used by Drachman (1971) to paralyze the hind limb of animals without systemic toxicity.

Schantz supplied Drachman and others with crystalline toxin, using at the University of Wisconsin's Food Research Institute the crystallization techniques developed at Fort Detrick by Lamanna and Duff. Schantz generously supplied us with botulinum toxin to do the experiments in animals on extraocular muscle, which were published in 1973 (Scott *et al., 1973)*. These showed that paralysis could be localized to the target muscle, its duration and depth controlled by dose, all without systemic side effects. We explicitly identified the potential utility of the drug in blepharospasm, and in other neurological conditions where ablation of unwanted tonus or movement was the goal.

The valuable clinical attributes of botulinum toxin include, first, its specificity. This restricts action to motor terminals, avoiding the sensory and other tissue effects that occur with drugs such as tetrodotoxin, saxitoxin, local anaesthetics, and other general membrane-active drugs. The long duration of action of botulinum toxin is its second most important feature. Once inside, the active part of the molecule is not substantially transported from the nerve terminal. The time from muscle paralysis until full recovery is typically 4-6 months. Schantz continued to send us crystalline toxin upon request with which we established pharmaceutical methods: buffering with human albumin, freeze drying, stability studies, determination of safe vial dosage, etc. Our application to the Food and Drug Administration (FDA) to issue an IND for clinical trials languished for years. We injected the first strabismus patient in 1977, 12 in 1978, and by 1982 we had injected the eye muscles for strabismus and nystagmus, the lid muscles for blespharospasm, hemifacial spasm, and lid retraction, the gastrocnemius and thigh adductors for spasm due to central nervous system disease, and the neck for torticollis. Others extended this application to spasmodic dysphonia, to other childhood spastic disorders ('cerebral palsy'), and to painful and debilitating limb muscle contracture following stroke or spinal disease. The drug was not patented by us, and the CAMR, Porton team, led by Melling, began a parallel development of type A toxin, using absorption purification in addition to chemical precipitation. Apart from dosage variation (see elsewhere in this volume), these products have similar effects. The contributions of over 200 clinical investigators rapidly expanded the knowledge base, subjecting the broad range of localized movement and localized tonic disorders to clinical trial.

Following approval of this drug by the FDA in December 1989, the widespread use of botulinum toxin has been seen in gastroenterology, cosmetic problems and tremor.These applications altogether give major impetus (and financial support!) to understanding basic structure, physiologic mechanisms and improvements of various toxin types.

For the future, reduction of antibody development with less antigen (purer toxin) should soon occur – we have shown that rabbits produce antibodies twice as fast to cumulative doses of one commercially available product as to another and combinations of types reduce the antigen load. Prolongation of effect is the major clinical need. A combination of types A + B (with different intracellular target proteins) is not more effective than each toxin alone, but other combinations may prolong effect. Do neurons recover by sprouting? – If so, then slowing this process will prolong recovery. Or, if sprouts retreat (as I believe) and the neuron recovers, perhaps it will be possible to prolong detoxification of toxin or to slow replacement of SNARE proteins. We are continuing to work on hybrid proteins with a more toxic moiety added to the specific receptor sequence. Extension of our knowledge of secretory mechanisms and their manipulation seems likely to be a major offspring of botulinum research.

References

Drachman DB (1971) Botulinum toxin as a tool fr research in the nervous system. In Simpson LL (ed) *Neuropoisons – Their Pathophysiological Action.* Plenum Press, New York; pp 325–347.

Gruesser OJ (1986) Die ersten systematischen Beschreibungen und tierexperimentellen Untersuchungen des Botulismus. *Sudhoffs Archiv* **70**, 167–87.

Scott AB, Rosenbaum AL and Collins CC (1973) Pharmacologic weakening of extraocular muscles. *Invest Ophthal* **12**, 924–27.

Botulinum toxin for strabismus

John Lee

Moorfields Eye Hospital, City Road, London EC1V 2PD

Keywords: Botulinum toxin, strabismus, nystagmus, oscillopsia

The therapeutic use of botulinum neurotoxin was first introduced by Alan Scott for strabismus and other disorders of ocular motility. Our group has used the agent therapeutically since 1982. This paper reports our practice and conclusions to date, with special attention to the indications for its use.

Introduction

The first report of any kind regarding this form of therapy was by Scott, Rosenbaum and Collins (1973), who reported some experiments on adult rhesus monkeys, in which under ketamine anaesthesia they had injected a variety of substances into the ocular muscles. The agents were DFP, (an anti-cholinesterase), α-bungarotoxin (a cobra venom), absolute alcohol and botulinum neurotoxin A. The botulinum toxin gave adequate alteration in ocular alignment without any significant systemic complications whereas none of the others had useful effects.

Alan Scott (1979) then reported his clinical experience in his first 19 patients and showed that this was a therapeutically viable technique without significant systemic toxicity and could be used in the management of a variety of types of strabismus. The principle is very simple. The 'over-acting' muscle is injected under electromyographic control with botulinum neurotoxin A. Two or so days later flaccid paralysis takes place and there is a change in ocular position. This change will last for a substantial period of time, typically months and recovery will then take place. If there is potential binocular fusion then the patient will usually regain the use of both eyes together and may make a full recovery. If, on the other hand, the patient has no binocular function, then it is likely that the deviation will recur and may be further treated, either surgically or with subsequent injections of toxin.

Method

The agent is delivered in an appropriate concentration in a volume of 0.1 ml of fresh aqueous isotonic solution, through a monopolar injection electrode under continuous electromyographic monitoring. Our current standard dose is 62.5 pg (2.5 European Dysport units) and we use this for all muscles. We have experimented with different concentrations and dosages but find that, although one can produce greater therapeutic effect by increasing the dose, one also increases the incidence of side-effects and have tended to settle upon this dosage as having the best balance of effects.

Anaesthesia is easily achieved, with 4 drops of 1% amethocaine and a drop of adrenaline (0.01%) administered just before starting the injection. Children may be treated by using intravenous ketamine 0.5 to 1mg per kg in addition, to topical local anaesthesia. Patients are seated in an appropriate reclinable chair, two electrodes (group, reference) are attached to the brow and connected to the monitoring equipment. We use fairly elaborate monitoring equipment with oscilloscope display but all that is essential is an audio amplifier and loudspeaker to allow the operator to judge the position of the needle. Such devices, battery powered, can be obtained or built relatively easily.

The electrode is insulated with epoxy resin right up to its tip and acts as a monopolar injection electrode. The muscle is engaged under direct vision with the patient looking away from that direction and then the patient is asked to look in the direction of action of the target muscle. The needle is advanced slowly down the muscle, listening all the while to the sound of the signal until this is maximal, at which point the injection is given. It is advisable to wait for a period of between thirty and forty seconds for the solution to diffuse into the muscle. The needle is then removed and the patient is then allowed to leave the clinic. If the patient has glasses of their own they are encouraged to wear them. If not, we place a pad on the injected eye to prevent any foreign body blowing

in while there is still corneal anaesthesia but no other follow up precautions are required. We review all new patients one or two weeks following the injection, however, patients with experience of the treatment only need to re-attend when they require a further injection.

We have seen no systemic complications of this treatment and none have been reported. This is not surprising because the dose is well below that required to produce any kind of significant systemic upset. Nevertheless there are minor complications and in our clinic these involve transient ptosis, transient hypertropia of the injected eye, subconjunctival haemorrhage and occasionally disorientation, past-pointing and diplopia. More major complications which have been reported include globe perforation and retrobulbar haemorrhage, both of which are extremely rare in experienced hands and have never occurred in our clinic.

Major Indications

The main indications for the use of botulinum toxin in strabismus, are (i) paralytic strabismus, especially sixth nerve palsy, (ii) as a diagnostic aid, (iii) cosmetic maintenance therapy and (iv) surgical over-corrections.

In addition, the agent is of considerable value in the management of cases of acquired nystagmus and oscillopsia.

Paralytic Strabismus

In paralytic strabismus the main value is in sixth nerve palsy, whether this is unilateral, bilateral, complete or partial. There are some selected cases of third nerve palsy in which the agent is of value but it is of little value in the majority of cases of fourth nerve palsy.

The *diagnostic* use of botulinum toxin by temporarily improving ocular alignment, permits distinction between partial and complete palsy and the detection of post traumatic disruption of fusion in patients who sustain substantial head injury and also have a cranial nerve palsy.

The *prophylactic* role is not yet proven. Although there have been a number of anecdotal studies reported in which a prophylactic role has been claimed, a randomised prospective controlled trial, (Lee *et al.*, 1994), in acute sixth nerve palsy has shown no real evidence of any effect of botulinum toxin on long term recovery, although patients certainly experience subjective benefit from the agent and we would not suggest that patients should not be treated.

There is no doubt that patients with established sixth nerve palsy may be *cured* with an injection of botulinum toxin. We have seen this effect on a number of occasions and would recommend in all cases of established sixth nerve palsy persisting more than six months after onset, it would be prudent to try the effects of botulinum toxin to see if the patient can be restored to normal binocular function by relief of established medial rectus contracture.

In patients who have poor general health or who have an inoperable intra-cranial tumour, one may use botulinum toxin on a *maintenance* basis so that single vision may be maintained without an uncomfortable head posture. Treatment can be repeated as often as necessary.

Botulinum toxin is of considerable value in the management of unrecovered sixth nerve palsy when used *in conjunction with surgery*. The problems of unrecovered sixth palsy are the absence of abducting force on the eye, the presence of medial rectus contracture and the risk of poor anterior segment blood supply. The surgical solution for this problem is complex because of risk of damage to the blood supply of the anterior ocular structures which is derived from the muscular vessels. Various solutions have been suggested for this. Our current practice is to perform an injection of botulinum toxin to the medial rectus muscle prior to surgery, followed by two muscle transposition surgery on the vertical recti. We and others (Fitzsimons *et al.*, 1988, Rosenbaum *et al.*, 1989) have shown that this is a highly effective procedure with a very low risk of anterior segment ischaemia, which also affords the possibility of subsequent exploration and recession of the medial rectus if the patient is still left with some limitation of his field of single vision following the primary procedure.

Selected cases of *third nerve palsy*, with relatively well preserved adduction, may be treated with botulinum toxin. Most cases of *fourth nerve palsy* do not gain from the use of toxin treatment and surgery is preferable in the majority of cases. *Multiple cranial nerve palsies*, in our clinic, usually have some combination of sixth and other cranial nerve palsy and can be managed as partial sixth nerve palsy.

Cosmetic Maintenance Therapy

The numerically most frequent general indication for the use of botulinum toxin in our clinic is for the management of adults or adolescents with cosmetically unsatisfactory deviations. There are two primary indications. One is simple aesthetic improvement and the other is the risk, raised by pre-operative orthoptic testing, of diplopia if the patient's eyes were straightened.

The commonest single indication for the use of botulinum toxin in our clinic is consecutive exotropia which amounts to around 20% of all our injections. These patients, who have frequently had many

operations, have no binocular function and yet have an aesthetic problem which they wish to have treated. They are extremely happy with this form of treatment and many of them have a substantial number of injections spread over a long period of time. We have recently reported (Lee, 1995) a series of patients treated with toxin as a long term therapy and have shown such patients are perfectly happy to continue on this line of treatment.

The use of pre-operative prism test to assess the risk of post-operative diplopia may be extremely misleading, as many patients will report such diplopia, yet do not have a problem when aligned long-term. It is now our practice to treat such patients with toxin to achieve temporary alignment. If diplopia is absent (the usual finding), the patient may be reassured that surgery will be equally unlikely to cause problems. Some patients note diplopia but are not inconvenienced by it. They can make a reasoned choice between such diplopia and improved cosmetic appearance.

Such *diagnostic* use of toxin can also be applied to patients with secondary strabismus such as in acquired aphakia, patients with head postures and patients with any type of dubious binocular function.

Surgical over-correction may also respond to botulinum toxin and, in particular, surgically over-corrected exotropia especially in children who respond extremely well to this agent. Such patients often have persistent diplopia after surgery and there is a real risk of loss of binocular function and/or amblyopia unless they receive fairly speedy therapy. Botulinum toxin temporarily paralyses the over-tightened medial rectus, allows the ocular position to become stabilised and nearly always avoids subsequent surgery.

Acquired Nystagmus and Oscillopsia

Adult patients with acquired nystagmus frequently complain bitterly of oscillopsia, which may be seriously disabling to them. Such patients may be offered symptomatic therapy with retro-bulbar injection of botulinum toxin, to reduce the amount of ocular movement and improve visual acuity (Ruben *et al.*, 1994). Patients have to understand that the treatment can only be given to one eye at a time and that the other eye will be occluded. They also may suffer ptosis after injection, although this is minimised by keeping the needle point well anterior in the muscle cone. In addition, ambulatory patients may be aware of loss of the normal vestibulo-ocular reflex eye movements.

Dosage is 10 times that required for one rectus muscle, ie 25 Dysport units in 1 ml. The best results are obtained in cases with oculo-palatal myoclonus and similar conditions with good potential visual acuity. Patients with advanced multiple sclerosis may try treatment but are often disappointed by results as they tend to have very poor vision due to repeated attacks or optic nerve demyelination.

Cumulative Clinic Experience

Between November 1982 and June 1996 we treated 3025 patients with the proportions of concomitant strabismus 78.2%, paralytic strabismus 13.7%, restrictive and muscular strabismus 13.7%, restrictive and muscular strabismus 6.9% and miscellaneous indications 1.2%.

The concomitant cases consisted of 915(38.7%) cases with esotropia (convergent squint), 1412 (59.7%) cases of exotropia (divergent squint) and 38 (1.6%) cases of hypotropia (vertical squint). The single most frequent indication for injection was consecutive exotropia, ie divergence of the eye in a non-binocular individual after childhood convergent squint. The proportion with this diagnosis was 21.3% of the entire group.

In the paralytic group of 414 cases, unilateral or bilateral sixth nerve palsy accounted for 333 cases, 80.4% of the group. The only other diagnoses that were notable were unilateral third nerve palsy (8%) and multiple cranial nerve palsy (4.8%). Restrictive and muscular types of squint accounted for 208 cases, with the two main indications being squint after retinal detachment surgery (35.6%) and dysthyroid ophthalmopathy (26%).

Conclusion

Advantages of botulinum toxin therapy are that it is a simple outpatient therapy in which patients gain understanding of their own condition and if the effects are not desired they wear off if no further action is taken. The *disadvantages* are that it is a frightening experience especially the first time it is done, it may well be somewhat painful if scar tissue is present and children require admission and ketamine anaesthesia.

The major problem, perhaps, is that this is an expensive therapy requiring a cost of £200 to open one vial of toxin and therefore rather difficult for the individual practitioner to justify for one or two patients. However, at our institution, as our colleagues have referred many patients, we treat on average 17-18 patients in each of our clinics, thereby minimising the unit cost.

We would therefore recommend the use of botulinum toxin as a therapeutic agent in strabismus for cases with an unpredictable surgical outcome, for cases with predicted post-operative diplopia, patients with sixth nerve palsy and patients with acquired nystagmus and oscillopsia.

We would not recommend its use in most types of childhood strabismus, in the management of fourth nerve palsy and in the management of accommodative deviations. Nevertheless, in selected patients it should be considered as the most useful new addition to our treatment since the introduction of strabismus surgery by Dieffenbach in 1893.

References

Fitzsimons R, Lee JP, Elston JS (1988) Treatment of sixth nerve palsy in adults with combined botulinum toxin chemo-denervation and surgery. *Ophthalmology*. **95**. 1535-1542.

Lee JP (1995) Long term use of botulinum toxin for adult strabismus. *Update on Strabismus and Pediatric Ophthalmology, ed. Lennerstrand*. pp 433-6.

Lee JP, Harris S, Cohen J, Cooper K, MacEwen C, Jones S. (1994) Results of a prospective randomised trial of botulinum toxin therapy in acute sixth nerve palsy. *J Ped Ophthalmol & Strabismus*. **31**. 283-6

Rosenbaum AL, Kushner BJ, Kirschen DL (1989) Vertical rectus muscle transpositlion and botulinum toxin (Oculinum) to medial rectus for abducens palsy. Arch Ophthalmol. **107**. 820-3

Ruben ST, Lee JP, O'Neill D, Dunlop I, Elston JS (1994) The use of botulinum toxin for treatment of acquired nystagmus and oscillopsia. *Ophthalmology*. **101**. 783-7.

Scott AB, Rosenbaum AL, Collins CC (1973) Pharmacologic weakening of eye muscles. *Invest Ophthalmol Vis Science*. **12**. 924-7.

Scott AB (1979) Botulinum toxin injection into extraocular muscles as an alternative to strabismus surgery. *Ophthalmology*. **87**. 1044-9.

Neurological indications for botulinum treatment

J.D. Speelman, †P.P. Devriese, M. Aramideh, J.W.M. Brans, *J.H.T.M. Koelman and *B.W. Ongerboer de Visser

Department of Neurology, *Division of Clinical Neurophysiology, †Department of E.N.T., Academic Medical Centre, University of Amsterdam, The Netherlands

keywords: botulinum, dystonia, electromyography, blepharospasm, spasmodic torticollis, hemifacial spasm

Since its introduction in 1983, the indications for botulinum toxin type A (BTA) in neurological disorders are increasing. In all types of focal or segmental dystonias, hemifacial spasm, spasticity, tremor, myoclonias, and muscle cramps, and even for tension headache, BTA has been applied. Controlled and follow up studies have been published for the approved indications: blepharospasm, cervical dystonia and hemifacial spasm. However, controversy still exists about various issues, such as application with or without EMG-guiding, single or multiple injection per muscle, site of injection, and dosage of BTA. Another point of importance is the assessment of the effects of BTA. Of much concern are the secondary non-responders, due to immunologic BTA resistance, especially in cervical dystonia, and other indications, who require high dosages of BTA. Surgical therapy for hemifacial spasm, blepharospasm or spasmodic torticollis may be considered in selected cases, but BTA-treatment remains the therapy of first choice.

In 1980 botulinum toxin type A (BTA) treatment was initiated in the Netherlands by the ophthalmologist Crone, inspired by the pioneering work of Scott (1980). The first injections were given by de Jong (ophthalmologist) and Devriese (otolaryngiologist) in 1981 in patients with diplopia, hemifacial spasm and blepharospasm. The neurological department in Amsterdam started in 1985 with the treatment of spasmodic torticollis and blepharospasm. Nowadays, in 26 hospitals spread over the Netherlands botulinum treatment is given for various neurological indications.

The main group of indications form the so-called dystonias, characterized by involuntary, sustained muscle contractions, frequently causing twisting and repetitive movements or abnormal postures. (Fahn *et al.*, 1987). The dystonias are divided in subtypes, according to the extension of the movement disorder: **focal**, involvement of only one body region, *i.e.* blepharospasm, spasmodic torticollis; **segmental**, two adjacent body regions, *i.e.* eyelids and oromandibular region, neck and arm; **generalized**, all body parts involved; **multifocal**, various regions, spread over the body. Due to the limitation of BTA dosages only focal or segmental disorders can be treated with BTA.

The neurological indications for botulinum treatment may be separated into three categories: **accepted**, registered officially for BTA treatment in at least three countries; **probable**, no official registration, but controlled studies, or several case reports available, indicating a favourable response; **questionable**, a single report is, or conflicting reports are available only (Table 1).

Based on the prevalence study in the USA (Nutt *et al.*, 1988) and estimation of clinical experiences, it is assumed that in the Netherlands with a population of 15×10^6 about 10,000 patients are eligible for BTA treatment (Table 2).

Technique of botulinum treatment

The freeze dried BTA is dissolved in sterile normal saline for injection. The dilution depends on the indication and dosage to be used. For most indications, the BTA is injected under concommittent electromyographic guiding, through a hollow needle with an isolated teflon coated shaft, but with a bare tip for the monopolar recording of the muscle action poten-

Correspondence: Dr. J.D. Speelman, Department of Neurology (H2-214), P.O.Box 22700, 1100 DE Amsterdam, The Netherlands. Phone: +31.20.566.4562; Fax: +31.20.697.1438.

Table 1. Neurological indications for BTA treatment

*Category**	*Indications*
accepted	blepharospasm, hemifacial spasm, spasmodic torticollis
possible	limb dystonia, oromandibular dystonia, spasmodic dysphonia, limb tremor, spasticity, palatum tremor, trismus, lingual dyskinesia, spasmodic dysphonia
questionable	pain management, f.e. tension headache, myalgia, tennis elbow

*For definition, see text.

Table 2. Estimations of the number of patients eligible for BTA treatment in the Netherlands

Indication	Prevalence[1]	Eligible for BTA treatment
blepharospasm	1500	1000
spasmodic torticollis	3000	2000
spasmodic dysphonia	1000	1000
oromandibular dystonia	1000	500
occupational cramp	1500	500
generalized dystonia	500	–
hemifacial spasm	3000	2000
spasticity	?	1000
secondary dystonias[2]	?	1000

[1] Prevalence of dystonias (Nutt *et al.*, 1988)
[2] Including cerebral palsy patients and drugs-induced dyskinesias

tials. In this way it can be verified whether the BTA is injected in the intended muscle.

So far, there is agreement that there is no difference in clinical use of both the two commercially available BTA products, Dysport (Speywood Pharmaceuticals Ltd) and Botox (Allergan Ltd). However, due to differences in the test procedures for the determination of the biological activity expressed in International Units (mouse LD 50), the International Units (I.U.) of both products are different in a ratio of respectively 3–6:1. We use the ratio of 4 I.U. Dysport equivalent to 1 I.U. Botox, based on our own dose finding study.

Controversies

Despite more than 10 years of BTA treatment for various neurological indications, a number of controversies still exist. In this paper a few points will be highlighted in the discussion on treatment of blepharospasm and spasmodic torticollis with regard to injection sites and the application of BTA with simultaneous electromyographic (EMG) guiding.

Blepharospasm is a condition in which involuntary closure of the eyelids causes a disabling impairment of vision, eventually leading to functional blindness. The disturbed eyelid closure may be caused by: A, an involuntary forceful closure of the eyelids, due to spasm of the orbicularis oculi muscle, the eyelid closing muscle; B, an inability of opening the eyelids or of keeping the eyelids open, due to a functional disturbance of the levator palpebrae superior muscle, the eyelid opening muscle; C, a combination of A and B. By means of synchronous EMG recordings from both muscles by small needle electrodes, the three types of abnormal eyelid closure can be differentiated (Aramideh *et al.*, 1994) and allows the prediction of the effect of BTA treatment: type A reacts very favourably, type C gives a less good response, and type B cannot be treated properly by BTA only, but needs additional provision in the form of orbicularis oculi crutches to keep the eyelids open.

Moreover, based on the EMG studies of the eyelid muscles, we could confirm the finding of the functional subdivision of the orbicularis oculi muscle in the three compartments, an orbital, a preseptal and a pretarsal part (Fig. 1). (Elston, 1992; Aramideh *et al.*, 1995).

It appeared that the pretarsal part of the orbicularis oculi muscle is of utmost importance in blepharospasm and has to be included in the injection sites of BTA. In our opinion:

1. BTA has to be applied in 5 sites, always including the pretarsal part of the orbicularis oculi muscle;
2. in the case of failure of BTA treatment, EMG has to be performed of the eyelid muscles, to analyze the involvement of the levator palpebrae sup. muscle.

Spasmodic torticollis. A focal form of dystonia involving neck muscles and resulting in abnormal posturing and/or involuntary movements of the head. Discussion is still going whether BTA has to be applied entirely under EMG-guiding in the different neck muscles involved. In our opinion, it is mandatory to use the EMG-guiding technique in order to be able to apply the BTA in the intended muscles. Even in superficial muscles it is sometimes difficult to adjust the point of the injecting needle in the muscle, especially in times of reinjections, if muscles are mostly still atrophic. (Speelman, 1995) As a consequence our dosages of BTA are low and we meet less side effects compared with reports in the literature. (Brans *et al.*, 1995)

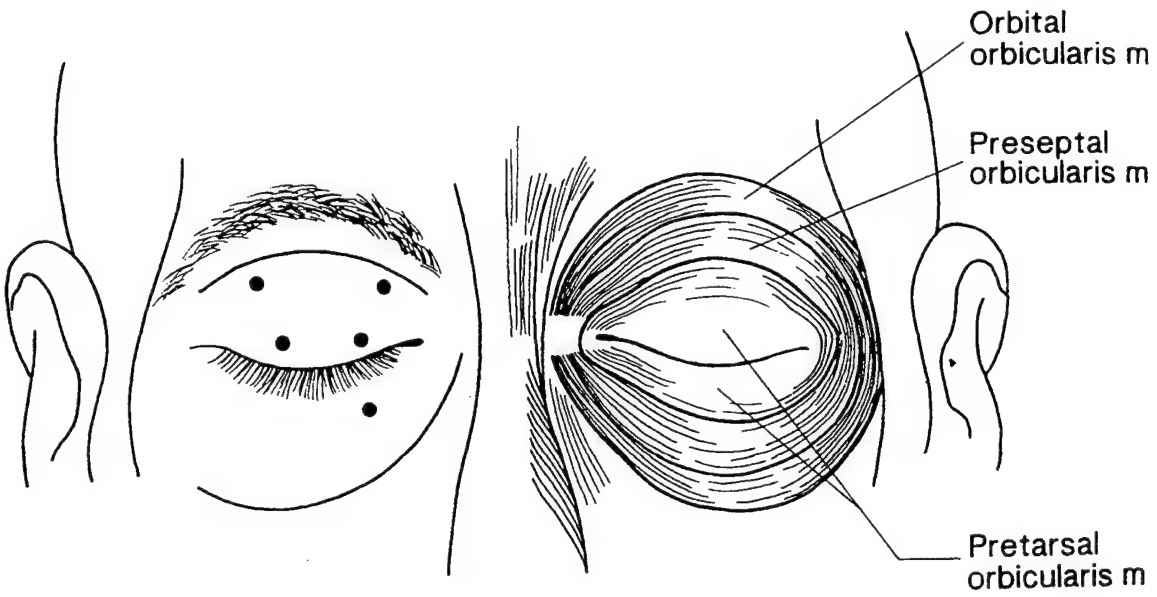


Figure 1. Subdivision of orbicularis oculi muscle and injection sites (•) for botulinum.

Non-responding to botulinum treatment

Three types can be discussed: a. primary non-responders; b. responders without clinical improvement; and c. secondary non-responders.

a. *Primary non-responders* are patients without any clinical effect by BTA application, neither muscular atrophy, nor EMG signs of de- and reinnervation. This is probably caused by inactivation of the BTA by antibodies.
b. *Responders without improvement.* Despite muscle atrophy and de- and reinnervation activity by EMG recording after BTA application, the patient demonstrates no clinical improvement or even deterioration. The following causes may play a role: Firstly, wrong selection of muscles or injections site(s),or the dosage of BTA is too low. Secondly, development of other symptoms, which may nullify the BTA effects, such as jerks or tremor, or non-injected muscles may become active, as has been observed in spasmodic torticollis. In these cases, it may sometimes be difficult to convince the patient to continue BTA treatment working towards a better result. Thirdly, wrong selection of patients, *i.e.* patients with blepharospasm, mainly due to impairment of the levator palpebrae superior muscle, or a non-dystonic spasmodic torticollis patient due to a fibrotic sternocleidomastoid muscle. (Brans *et al.*, 1996).
c. *Secondary non-responders*, patients who initially show a clear symptomatic improvement which cannot be reproduced by repeated injections. In a number of patients antibodies against BTA can be demonstrated. Especially liable are patients who require high dosages of BTA, applied within short intervals, or who receive regularly booster injections for reinforcing the BTA effects. Sometimes it appeared that the non-responding is due to inappropriate BTA application, *i.e.* without EMG guiding of the injection.

Botulinum treatment or surgery

This question will be discussed for the three 'accepted' neurological indications, hemifacial spasm, blepharospasm and spasmodic torticollis.

Hemifacial spasm. The clinical picture is involuntary spasm of the unilateral facial musculature. The most common cause is activation of the facial nerve root near the brain stem, due to compression by a vascular structure. Microvascular decompression is a surgical procedure in which the facial nerve root is freed from the compressing structure. (Janetta, 1981) Surgical risks involve damage to the facial nerve, mostly transient, and permanent deafness due to damage of the vascularisation of the acoustic nerve in about 10% of the operations.

Surgical treatment may be considered in a relatively young patient, who has little or no response to botulinum treatment, or who refuses a lifelong injection treatment with BTA. An absolute contraindication is a severe loss of hearing or deafness at

the contralateral ear. Older age is a relative contraindication for surgery due to additional surgical risks.

Blepharospasm. Two surgical techniques are used for blepharospasm, myectomy according to Anderson, and peripheral nerve denervation of the orbicularis oculi muscle. Both techniques have a relatively high rate of recurrence, and may be accomplished by side effects. (Frueh *et al.*, 1976; Anderson, 1984) Botulinum treatment is the first choice therapy, and we do not discuss surgical options with the patients, except when they ask for alternative treatments. Sometimes, anticholinergics, or Clonazepam, may lead to some clinical improvement. In secondary non-responders with mainly orbicularis oculi muscle involvement, surgery may be performed, we then prefer myectomy. But in the case of little or no improvement, and mainly levator palpebrae sup. muscle involvement, we do not recommend surgical therapy.

Spasmodic torticollis. BTA treatment is the first choice, combined with physical therapy. In the case of secondary non-responders, a selective peripheral nerve denervation can be considered. This surgical technique has been developed by Bertrand, and a denervation of involved muscles is performed, with sparing of the trapezius and lateral neck muscles. (Bertrand *et al.*, 1988) Another operation indication may be a denervation of an involved sternocleidomastoid muscle, in the case this always need BTA application. It is a simple surgical procedure, and diminishes the dosage of BTA.

Epilogue

In the Netherlands we annually organize a Botulinum workshop for neurologists involved in BTA treatment. There we discuss indications for BTA treatment, techniques, patient selection, results and failures. In this way, we try to reach consensus about various controversies and questions related to botulinum treatment in our country.

References

Anderson RL (1984) Periorbital blepharospasm surgery. In Jakobiec, FA and Sigelman, J. (eds): *Advanced techniques in ocular surgery.* Philadelphia: WB Saunders; pp 572–588.

Aramideh M, Ongerboer de Visser BW, Devriese PP, Bour LJ and Speelman JD (1994) Electromyographic features of levator palpebrae superioris and orbicularis oculi muscles in blepharospasm *Brain* **117**, 27–38.

Aramideh M, Ongerboer de Visser BW, Brans JWM, Koelman JHTM and Speelman JD (1995) Pretarsal application of botulinum toxin for treatment of blepharospasm *JNNP* **59**, 309–311.

Bertrand CM, Molina-Negro P, Bouvier G *et al.* (1988) Observations and analytical results in 111 cases of spasmodic torticollis after selective denervation *Appl Neurophysiol* **50**, 319–323.

Brans JWM, de Boer IP, Aramideh M, Ongerboer de Visser BW and Speelman JD (1995) Botulinum toxin in cervical dystonia: low dosage with electromyographic guidance *J Neurol* **242**, 529–534.

Brans JWM, Aramideh M, Bosch DA and Speelman JD (1996) Late presentation of congenital muscular torticollis: a non-dystonic cause of torticollis *J Neurol* **243**, 354–356.

Elston JS (1992) A new variant of blepharospasm *J Neurol Neurosurg Psychiatry* **55**, 369–371.

Fahn S, Marsden CD and Calne DB (1987) Classification and investigation of dystonia. In Marsden, CA and Fahn, S (eds): *Movement disorders 2.* London: Butterworths; pp 332–358.

Frueh BR, Callahan A, Dortzbach RK *et al.* (1976) The effects of differential section of the 7th nerve on patients with intractable blepharospasm *Trans Am Acad Ophthalmol Otolaryngol* **81**, 595–602.

Janetta PJ (1981) Hemifacial spasm. In Samii M and Janetta PJ (eds): Berlin: Springer Verlag; pp 484–493.

Nutt JG, Muenter MA, Aronson A, Leonard T and Melton LJ (1988) Epidemiology of focal and generalized dystonia in Rochester, Minnesota *Mov Disord* **3**, 188–194.

Scott AB (1980) Botulinum toxin injections to eye muscles to correct strabismus *J Am Ophthalmol Soc* **79**, 734–770.

Speelman JD and Brans JWM (1995) Cervical dystonia and botulinum treatment. Is electromyographyc guidance necessary? *Mov Disord* **10**, 802.

Novel therapeutic agents

Keith A. Foster

CAMR, Porton Down, Salisbury, Wiltshire SP4 0JG

Keywords: Drug delivery, Endopeptidase, Exocytosis, Heavy-chain, Light-chain, Receptor, SNAP-25, SNARE, Syntaxin, VAMP

It is possible to allocate discrete biological functions to individual domains of the clostridial neurotoxin polypeptide structure. These separate components of toxin function offer opportunities for developing novel therapeutics.

Clostridial neurotoxins are dichain protein toxins consisting of a 100 kDa heavy chain (HC) and a 55 kDa light chain (LC) linked by a disulphide bond and strong non covalent interactions (DasGupta, 1989). HC is susceptible to limited proteolysis to yield two fragments of approximately equal size, the carboxy-terminal half, termed H_C, and the amino-terminal half, termed H_N. The mechanism of action of the neurotoxins involves three distinct phases, binding, internalisation and inhibition of neurosecretion (Simpson, 1993), and H_C, H_N and LC appear to represent functional domains responsible for the different stages. This discrete domain structure offers the opportunity for specific therapeutic uses based upon the component functions of neurotoxin action.

The Heavy Chain

The HC is responsible for the delivery and the specific binding of neurotoxin to the presynaptic membrane of the motorneuron and for the entry of the toxin into the neuron. Initially toxin binds to ecto-acceptors located on the neuronal surface with subsequent internalisation of the toxin into an endosomal compartment. The identity of the neuronal receptors for the clostridial neurotoxins remains to be established. It has recently been reported that synaptotagmin associated with gangliosides G_{T1b} and G_{D1a} is a neuronal binding site for BoNT/B (Nishiki *et al.*, 1996; Kozaki *et al.*, these proceedings). Competition studies have shown that types A and B do not share the same receptor, whilst types A and E do (Sellin, 1987). BoNT/F would appear to bind to a further distinct category of receptor (Wadsworth *et al.*, 1990). Tetanus toxin binds to a receptor which is involved in the subsequent retrograde axonal transport of that toxin (Morris *et al.*, 1980). It has recently been proposed that BoNT/D binds to a receptor absent from human motorneurons, and that this explains the lack of human botulism caused by BoNT/D (Simpson, 1995). Information regarding the binding sites for BoNT/ C_1 and /G is not yet available. There are, therefore, at least five categories of ecto-acceptor for the clostridial neurotoxins.

At least two populations of binding sites have been identified on neuronal membranes (Evans *et al.*, 1986): a small population of high affinity sites (K_d less than 1 nM) and a larger population of low affinity sites (K_d greater than 10 nM). Purified HC binds to synaptic membranes and is able to compete with native toxin for binding. Removal of H_C abolishes the ability to bind (Shone *et al.*, 1985). These findings indicate that the C-terminal portion of the HC is important for binding to the cell surface acceptors. This portion of tetanus neurotoxin also retains the ability to undergo retrograde axonal transport (Morris *et al.*, 1980). Daniels-Holgate and Dolly (1996) have reported that a proportion of the high affinity binding of BoNT/A is not antagonised by HC and that this represents the binding responsible for intoxication.

Following binding to its receptor neurotoxin is internalised by endocytosis. Acidification of the endosome is thought to induce a conformational change in the neurotoxin which enables insertion of a portion of the molecule into the endosomal membrane and translocation across the membrane and into the cytosol. It is believed that the H_N domain is responsible for this translocation activity

Correspondence: Keith A. Foster, Centre for Applied Microbiology and Research, Porton Down, Salisbury. SP4 OJG, UK. Tel: (44) 1980 612630; Fax: (44) 1980 610848.

(DasGupta, 1994). Acid-induced pore formation has been demonstrated using lipid vesicles with neurotoxin, HC, LH_N and H_N but not with LC (Shone *et al.*, 1987). Pore formation is believed to relate to the translocation function of the toxin but the exact mechanism is not yet understood (Montecucco & Papini, 1995).

The properties of the HC of clostridial neurotoxins mean that they offer a number of opportunities for selective macromolecular drug delivery to the nervous system. Tetanus neurotoxin HC offers opportunities for delivery to the central nervous system, whilst botulinum neurotoxins would be more appropriate for delivery to the peripheral nervous system. Further options for tailoring drug delivery are offered by the fact that different fragments of the neurotoxin display different binding characteristics. The observation that only intact neurotoxin binds in a manner able to result in inhibition of acetylcholine release (Daniels-Holgate and Dolly, 1996) suggests that intact neurotoxin would be most appropriate for delivery of agents targeted at the cholinergic system. Methods have been described for producing non-toxic neurotoxins based upon mutated recombinant LC for this purpose (Zhou *et al.*, 1995). HC is also able to bind and internalise into neuronal cells, however, and may be preferred, given its smaller size and inherent lack of toxicity, where non-cholinergic neurons are the target. The H_C fragment also binds to receptors on neuronal cells, but is probably unable to access the cell interior following endocytosis, a property that may offer another delivery strategy. Figueiredo and colleagues (these proceedings) have demonstrated the ability of the H_C fragment of tetanus neurotoxin to deliver superoxide dismutase applied at the periphery to the CNS.

Given the small numbers of molecules that are likely to be delivered to neuronal cells using vectors derived from the clostridial neurotoxins the types of application where this would be a suitable strategy are ones involving an amplification factor. Amongst the possible candidates for delivery are imaging agents, growth factors, hormones, enzymes and genes. The issues of protein load and chronic dosing also need to be considered given the potential immune response to a large non-host protein. Superoxide dismutase has been investigated as one candidate enzyme for delivery (Figueiredo *et al.*, these proceedings). This could have clinical utility in conditions of neuronal free radical toxicity, for example stroke and trauma. There is also evidence for superoxide dismutase deficiency in some forms of motor-neuron disease, including ALS. Another therapeutic problem for which a neurotoxin derived agent may represent the solution is the delivery of neurotrophins to the CNS. In this instance the subcellular site of delivery would be a crucial factor for success. To date there are no reported clinical applications of the clostridial neurotoxins or their derivatives as drug delivery vectors.

The Light Chain

If membrane binding and internalisation are bypassed through permeabilization or liposomal delivery, only the LC is required for intracellular inhibition of neurosecretion in vertebrates (Bittner *et al.*, 1989). The LC of clostridial neurotoxins are zinc-endopeptidases with specific substrate proteins, VAMP, syntaxin and SNAP-25, that are essential components of a neurosecretory complex responsible for the docking and fusion of synaptic vesicles with the presynaptic membrane (Sollner *et al.*, 1993). Isoforms of VAMP and syntaxin are present in most cells, where they are involved in a wide variety of vesicle dependent functions, and although SNAP-25 has not been identified in cells other than neuroendocrine, a homologue, SNAP-23, has been reported in non-neuronal cells (Ravichandran *et al.*, 1996). Given that the neurotoxin substrates are so widely distributed and fundamental to the process of vesicle fusion, proteolytic cleavage by neurotoxin could affect a range of vesicle-dependent processes.

It has long been recognised that a variety of neuronal secretions are sensitive to inhibition by clostridial neurotoxins, including both classical neurotransmitters and neuropeptides (Ashton *et al.*, 1993). Similarly it is known that exocytosis of large dense-core vesicles from chromaffin cells is inhibited by the clostridial neurotoxins, and that inhibition correlates closely with cleavage of the relevant substrate proteins (Lawrence *et al.*, 1996, Foran *et al.*, 1996). Insulin secretion from an insulinoma cell line, HIT-15, is sensitive to inhibition by both BoNT/A and /B, and inhibition of secretion is accompanied by cleavage of substrate protein (Boyd *et al.*, 1995). Similar results have been reported for insulin secretion from pancreatic islets (Sadoul *et al.*, 1995). ACTH release from AtT20 cells, has been demonstrated to be sensitive to inhibition by the LC of BoNT/A (Aguado *et al.*, these proceedings). In addition to neuronal and endocrine secretion, exocrine secretion is also sensitive to the neurotoxins, Fujita-Yoshigaki *et al.* (1996) reported that BoNT/B inhibited amylase release from permeabilised parotid acinar cells.

Not only secretory cells possess neurotoxin substrates; it is now becoming apparent that the clostridial neurotoxins affect the fusion of recycling vesicles, thereby modifying the expression of integral membrane proteins at the cell surface. The first such report was that tetanus toxin-mediated cleavage of VAMP in CHO cells impaired exocytosis of transfer-

rin receptor-containing vesicles (Galli *et al.*, 1994). Tetanus toxin inhibits fusion of endosomes containing aquaporin collecting duct water channel protein (Jo *et al.*, 1995). Tetanus toxin, and BoNT/F, also inhibits the incorporation of vesicular stomatitis virus spike glycoprotein into the basolateral membranes of MDCK cells (Ikonen *et al.*, 1995). Insertion of influenza virus haemagglutinin into the apical membrane was unaffected by toxin, suggesting that there may be a spatial element to the involvement of the neurotoxin substrate proteins in vesicle movement. In adipocytes, insulin-stimulated translocation of the glucose transporter GLUT4 is inhibited by BoNT/B (Tamori *et al.*, 1996).

Thus, the neurotoxin substrates are essential components of a fundamental and widely distributed vesicle fusion mechanism and the LCs of clostridial neurotoxins have the potential to modulate a variety of regulated exocytotic processes, both secretory and membrane protein cycling. As such the LC of the clostridial neurotoxins is able to modulate regulated exocytosis and, potentially, could be used as the basis of therapeutic agents for clinical pathologies involving inappropriate exocytotic fusion events.

References

Ashton AC, de Paiva AM, Poulain B, Tauc L & Dolly JO (1993) Factors underlying the characteristic inhibition of the neuronal release of transmitters by tetanus and various botulinum toxins. In DasGupta BR (ed): *Botulinum and Tetanus Neurotoxins: Neurotransmission and Biomedical Aspects,* Plenum Publishing Corp, New York, pp 191–213.

Bittner MA, DasGupta BR & Holz RW (1989) Isolated light chains of botulinum neurotoxins inhibit exocytosis. *J Biol Chem* **264**, 10354–10360.

Boyd RS, Duggan MJ, Shone CC & Foster KA (1995) The effect of botulinum neurotoxins on the release of insulin from the insulinoma cell lines HIT-15 and RINm5F. *J Biol Chem* **270**, 18216–18218.

Daniels-Holgate PU & Dolly JO (1996) Productive and non-productive binding of botulinum neurotoxin A to motor nerve endings are distinguished by its heavy chain. *J Neurosci Res* **44**, 263–271.

DasGupta BR (1989) The structure of botulinum neurotoxin. In Simpson LL (ed): *Botulinum Neurotoxins and Tetanus Toxin*, Academic Press, New York; pp 53–67.

DasGupta BR (1994) Structures of botulinum neurotoxin, its functional domains, and perspectives on the crystalline type A toxin. In Jankovic J & Heller M (eds): *Therapy with Botulinum Toxin*, Marcel Dekker Inc., New York; pp 15–39

Evans DM, Williams RS, Shone CC, Hambleton P, Melling J & Dolly JO (1986) Botulinum neurotoxin type B; its purification, radioiodination and interaction with rat-brain synaptosomal membranes. *Eur J Biochem* **154**, 409–416.

Foran P, Lawrence GW, Shone CC, Foster KA & Dolly JO (1996) Botulinum neurotoxin C1 cleaves both syntaxin and SNAP-25 in intact and permeabilized chromaffin cells: correlation with its blockade of catecholamine release. *Biochemistry* **35**, 2630–2636.

Fujita-Yoshigaki J, Dohke Y, Hara-Yokoyama M, Kamata Y, Kozaki S, Furuyama S & Sugiya H (1996) Vesicle-associated membrane protein 2 is essential for cAMP-regulated exocytosis in rat parotid acinar cells. *J Biol Chem* **271**, 13130–13134.

Galli T, Chilcote T, Mundigl O, Binz T, Neimann H & De Camilli P (1994) Tetanus toxin-mediated cleavage of cellubrevin impairs exocytosis of transferrin receptor-containing vesicles in CHO cells. *J Cell Biol* **125**, 1015–1023.

Ikonen E, Tagaya M, Ullrich O, Montecucco C & Simons K (1995) Different requirements for NSF, SNAP and Rab proteins in apical and basolateral transport in MDCK cells. *Cell* **81**, 571–580.

Jo I, Harris HW, Amendt-Raduege AM, Majewski RR & Hammond TG (1995) Rat kidney papilla contains abundant synaptobrevin protein that participates in the fusion of antidiuretic hormone-regulated water channel-containing endosomes *in vitro*. *Proc Natl Acad Sci USA* **92**, 1876–1880.

Lawrence GW, Foran P & Dolly JO (1996) Distinct exocytotic response of intact and permeabilised chromaffin cells after cleavage of the 25-kDa synaptosomal -associated protein (SNAP-25) or synaptobrevin by botulinum toxin A or B. *Eur J Biochem* **236**, 877–886.

Montecucco C & Papini E (1995) Cell penetration of bacterial protein toxins. *Trends Microbiol* **3**, 165–167.

Morris NP, Consiglio E, Kohn LD, Habig WH, Hardegree MC & Helting T (1980) Interaction of fragments B and C of tetanus toxin with neural and thyroid membranes and with gangliosides. *J Biol Chem* **255**, 6071–6076

Nishiki T, Tokuyama Y, Kamata Y, Nemoto Y, Yoshida A, Sato K, Sekiguchi M, Takahashi M & Kozaki S (1996) The high-affinity binding of *Clostridium botulinum* type B neurotoxin to synaptotagmin II associated with gangliosides G_{T1b}/G_{D1a} *FEBS Lett* **378**, 253–257

Ravichandran V, Chawla A & Roche PA (1996) Identification of a novel syntaxin- and synaptobrevin/VAMP-binding protein, SNAP-23, expressed in non-neuronal tissues. *J Biol Chem* **271**, 13300–13303.

Sadoul K, Lang J, Montecucco C, Weller U, Regazzi R, Catsicas S, Wollheim CB & Halban PA (1995) SNAP-25 is expressed in islets of langerhans and is involved in insulin release. *J Cell Biol* **128**, 1019–1028.

Sellin LC (1987) Botulinum toxin and the blockade of transmitter release. *Asia Pacific J Pharmacol* **2**, 203–222

Shone CC, Hambleton PH & Melling J (1985) Inactivation of *Clostridium botulinum* type A neurotoxin by trypsin and purification of two tryptic fragments *Eur J Biochem* **151**, 75–82.

Shone CC, Hambleton P & Melling J (1987) A 50-kDa fragment from the NH_2-terminus of the heavy subunit of *Clostridium botulinum* type A neurotoxin forms channels in lipid vesicles *Eur J Biochem* **167**, 175–180.

Simpson LL (1993) Current concepts on the mechanism of action of clostridial neurotoxins. In DasGupta BR (ed): *Botulinum and Tetanus Neurotoxins*, Plenum, New York; pp 5–15.

Simpson LL (1995) The structure and mechanism of action of botulinum neurotoxin. *Movement Disorders* **10**, 362.

Sollner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P & Rothman JE (1993) SNAP receptors implicated in vesicle targeting and fusion. *Nature* **362**, 318–324.

Tamori Y, Hashiramoto M, Araki S, Kamata Y, Takahashi M, Kozaki S & Kasuga M (1996) Cleavage of vesicle-associated membrane protein (VAMP)-2 and cellubrevin on GLUT4-containing vesicles inhibits the translocation of GLUT4 in 3T3-L1 adipocytes. *Biochem Biophys Res Comm* **220**, 740–745.

Wadsworth JD, Desai M, Tranter HS, King HJ, Hambleton P, Melling J, Dolly JO & Shone CC (1990) Botulinum type F neurotoxin. Large-scale purification and characterization of its binding to rat cerebrocortical synaptosomes. *Biochem J* **268**, 123–128.

Zhou L, de Paiva A, Liu D, Aoki R & Dolly JO (1995) Expression and purification of the light chain of botulinum neurotoxin A: a single mutation abolishes its cleavage of SNAP-25 and neurotoxicity after reconstitution with the heavy chain. *Biochemistry* **34**, 15175–15181.

Clostridal neurotoxins therapy: the future

John Elston

Oxford Eye Hospital, Radcliffe Infirmary NHS Trust, Woodstock Road, Oxford OX2 6HE, UK

Keywords: Botulinum Toxin, Dystonia, Spasticity, Tremor, Non Responders

In the light of the pattern of development of indications for botulinum toxin treatment over the past decade, the prospects for future development are considered. Four areas for progress are identified. Firstly, novel clinical indications for botulinum toxin treatment. Secondly, modification of the present treatment technique to increase efficacy. Thirdly, possible means of prolonging the period of benefit, and fourthly, new developments that may help patients responding poorly or not at all to this treatment.

Botulinum toxin treatment was originally developed for the management of strabismus. The aim of treatment is to alter the balance between antagonistic extra ocular muscles by selective muscle weakening, thereby changing ocular alignment. The indications and technique have been progressively refined and the treatment established as a valuable part of strabismus management. However, although there are a number of absolute indications for botulinum toxin treatment in strabismus (particularly paralytic strabismus), in most cases surgical alternatives exist. By contrast before the introduction of botulinum toxin treatment the management of diseases characterised by excessive unwanted muscle activity was very disappointing. These conditions are due to a variety of central nervous system disorders and manifest principally as dystonia, spasticity and tremor.

DYSTONIA is a disorder of the extra pyramidal system producing sustained involuntary muscle contractions. Depending on the part of the body affected there may be twisting, repetitive movements or abnormal posture. Prior to the introduction of botulinum toxin, treatment for focal dystonia (i.e. that restricted to a particular area of the body) was with systemic drugs. A large variety of agents with different modes of action were tried with very little objective evidence of benefit and often unpleasant side effects.

SPASTICITY is a feature of a variety of chronic neurological conditions. It is characterised by excessive muscle tone and involuntary contractions, due to release of descending (pyramidal) inhibition. Spasticity is often accompanied by pain with reduction of movement and function as well as spasms. Conventional treatment includes attempts at permanent surgical reduction of the innervation of the muscles.

TREMOR is a rhythmic oscillatory movement produced by alternating or synchronous contraction of antagonist muscles. Systemic drug treatment produces little relief.

The introduction and development of botulinum toxin treatment for these conditions over the past fifteen years has produced enormous benefits, principally for the individual patients concerned. It has also provided an opportunity for doctors to study relatively large numbers of patients with rare disorders and gain a better understanding of the pathophysiology and clinical characteristics of the disorders.

The clinical literature on botulinum toxin treatment of these conditions began to appear in the early 1980's, progressively increasing to reach a peak in the early 1990's. The treatments are now well established and the publication rate is declining. What does the future hold?

I will approach this question by asking four other questions. These are:

- Are there any other clinical conditions for which botulinum toxin treatment may be useful?
- Are there any modifications of the existing treatment technique that may increase the efficacy?
- Is there any means of prolonging the period of benefit from treatment?
- Is there any way of helping the sub group of patients who do not respond to botulinum toxin treatment?

Other clinical conditions for which botulinum toxin may be useful

Botulinum toxin treatment was originally developed by ophthalmologists then by neurologists. The potential benefit of focal denervation for the conditions encountered in these disciplines has been thoroughly explored. Other specialties, such as otolaryngology, urology and maxillofacial surgery have researched possibilities.

There is potential for further investigation in a number of specialities. In plastic and cosmetic surgery, inappropriate muscle tone or contraction may contribute to tissue distortion or even scarring which could be relieved by focal muscle weakening. In orthopaedics and sports medicine secondary muscle spasm may cause pain and secondary changes in posture or function that could cause continuing disability. Focal muscle weakening may be useful. This also applies in rehabilitation medicine, and intractable muscle spasm contributes to some pain syndromes. Pain relief specialists may find botulinum toxin a useful adjunct to treatment.

Modification of the technique to increase efficacy

Electromyography (emg) has been helpful in selecting muscles to be treated, for example in cervical dystonia, and blepharospasm. Lower doses can be used if placement is accurate. This may, in some conditions, avoid the development of antibodies, and generally means that side effects are reduced.

The expertise required to master a new technique can only be acquired at a referral centre. For many patients the travel and inconvenience involved may be considerable, and in the future interested primary care providers (for example general practitioners) may be able to treat individual patients. Self administration of the drug is a possibility if appropriate treatment kits are available.

Prolongation of benefit

It is established that the different serotypes of botulinum toxin bind selectively to specific receptors on the motor nerve terminal. Moreover, after internalisation the intra cellular action, whereby neuro transmitter exocytosis is blocked, is by enzymatic cleavage of different protein substrates (see Foster, K. in these proceedings) for each serotype. Individual toxins damage different components of the system. Combinations of botulinum toxin serotypes (for example A and F) may lead to a more profound disruption of the neurotransmitter exocytosis mechanism which would take longer to recover.

An alternative or parallel development would be to use evolving techniques of molecular biology engineering to produce a botulinum toxin molecule less susceptible to metabolism or elimination. Such techniques could also produce chimeric molecules, whereby the binding potential of, for example, type A could be combined with the intra cellular properties of e.g. type B. Alternatively the binding properties of the H chain of any stereotype could be used to internalise a substance that prevents or delays recovery from exocytosis block.

Different skeletal muscle types have different botulinum toxin receptor densities. In order to achieve the longest period of benefit at the lowest dose possible the stereotype used in treatment could be tailored to the muscle type.

Non responders

Primary non response to botulinum toxin treatment may be defined as a failure to benefit symptomatically or functionally despite adequate muscle weakness produced by botulinum injection. It occurs in ten to fifteen per cent of patients with focal dystonia, and is probably an indication of the severity of the disorder of muscle function in those individuals. More research is needed to characterise and identify these individuals and develop alternative treatments.

Secondary non response occurs when after a period of benefit from treatment further botulinum toxin injections failed to provided symptomatic relief. It may be due to the underlying disease process worsening to develop the characteristics of a primary non response. Secondary non response can also be due to antibodies to botulinum toxin. It may be possible to prevent the development of antibodies in patients requiring large doses of botulinum toxin by using a sequence of different serotypes. For example a patient could be treated in sequence with serotype A - then B - then F - and then A - B - F etc. If antibodies develop to an individual serotype a different one can be used. The most highly toxic serotype should be used to minimise antigen load.

Conclusions

The future of botulinum toxin treatment in medicine is secure. In a short period it has established an important role, particularly in the management of previously highly refractory disorders of excessive muscle activity. These conditions require long term management with repeated treatment. The treatment

techniques will continue to be refined and made more acceptable to the individual patients.

One of the most rewarding aspects of being involved in the development of the therapeutic applications of botulinum toxin over the past years has been the opportunity to collaborate and exchange ideas with biochemists and other scientists working with botulinum toxin. This collaboration must be encouraged to continue to produce further improvements in therapy.

Session 6

Structure, Action and as Probes III

Distinct contributions to exocytosis of two adjacent C-terminal regions of SNAP-25 unveiled by botulinum toxins A and E

J. Oliver Dolly, Gary W. Lawrence and Patrick Foran

Department of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7 2AY, U.K.

Types A and E botulinum neurotoxin (BoNT) are Zn^{2+}-requiring endoproteases which cleave 9 and 26 residues, respectively, from the C-terminus of synaptosomal-associated protein of Mr = 25 kDa (SNAP-25). Involvement of SNAP-25 in the exocytosis of large dense-core vesicles in bovine adreno-chromaffin cells was examined by measuring cleavage of SNAP-25 in relation to the levels of Ca^{2+}- evoked catecholamine release from cells exposed to BoNT/A or /E, either before or after digitonin-permeabilisation. The dose-dependency of inhibition of exocytosis correlated closely with the extent of SNAP-25 cleavage in cells permeabilised and then treated with BoNT/E. In intact cells exposed to 66 nM BoNT/A, virtually all of the SNAP-25 was truncated, accompanied by a near-complete inhibition of exocytosis; however, after their permeabilisation a significant level of secretion was recorded upon Ca^{2+}- stimulation. Importantly, this BoNT/A-resistant release from the permeabilised cells was dramatically lowered by subsequently adding BoNT/E which further truncated the SNAP-25 fragment that had been produced earlier by BoNT/A. Moreover, inclusion of anti-SNAP-25 IgG during the permeabilisation resulted in a reduction in the BoNT/A-insensitive exocytosis, reaffirming that the latter involves SNAP-25 lacking the C-terminal 9 residues. When permeabilised cells were exposed to either neurotoxin, both blocked MgATP-dependent secretion but only BoNT/E attenuated the energy-independent phase. These distinct inhibitory effects of the two neurotoxins demonstrate that residues 197-205 at the C-terminus of SNAP-25 are absolutely essential for exocytosis from intact cells whereas even after their removal a significant proportion of the exocytotic response can be elicited from permeabilised cells, but this is reliant on amino acids 180-196. Moreover, the latter but not residues 197-205 are implicated in a late, MgATP-independent step of exocytosis which is blocked by BoNT/E but non-susceptible to BoNT/A.

Seven immunologically-distinct serotypes of botulinum neurotoxin (BoNT; A-G), Zn^{2+}-endoproteases produced by *Clostridium botulinum*, inhibit the synaptosomal release of all neurotransmitters including neuropeptides (Ashton *et al.*, 1988; McMahon *et al.*, 1992), as well as secretion from permeabilised neuroendocrine cells (Bittner *et al.*, 1989; McInnes and Dolly, 1990), if delivered intracellularly. Their selective endoproteolytic activities were first demonstrated using synaptic preparations (for review, see Montecucco and Schiavo, 1995; Dolly *et al.*, 1994); BoNT/B, D, F, and G cleave synaptobrevin (also known as vesicle-associated membrane-protein) and a homologue called cellubrevin; A and E truncate synaptosomal-associated protein of Mr = 25 kDa (SNAP-25) and C proteolyses syntaxin and SNAP-25 (Foran *et al.*, 1996). As expression of the neurotoxins' substrates in neuroendocrine cells has been detected (Hodel *et al.*, 1994; Roth and Burgoyne, 1994), we investigated the importance of each of these three proteins in the exocytosis of large dense-core granules from adrenal medullary chromaffin cells maintained in culture (Foran *et al.*, 1995, 1996, Lawrence *et al.*, 1996, 1997). This contribution will concentrate on the role in exocytosis of two adjacent C-terminal regions of SNAP-25.

Results

Both BoNT/A and E cleave SNAP-25 and inhibit triggered secretion of catecholamines from chromaffin cells.

Evoked catecholamine secretion from *intact* chromaffin cells was blocked following their exposure for 24 h to BoNT/A in a low ionic strength medium (Fig. 1A). This was accompanied by a dose-dependent and selective cleavage of SNAP-25 (Fig 1B), as monitored

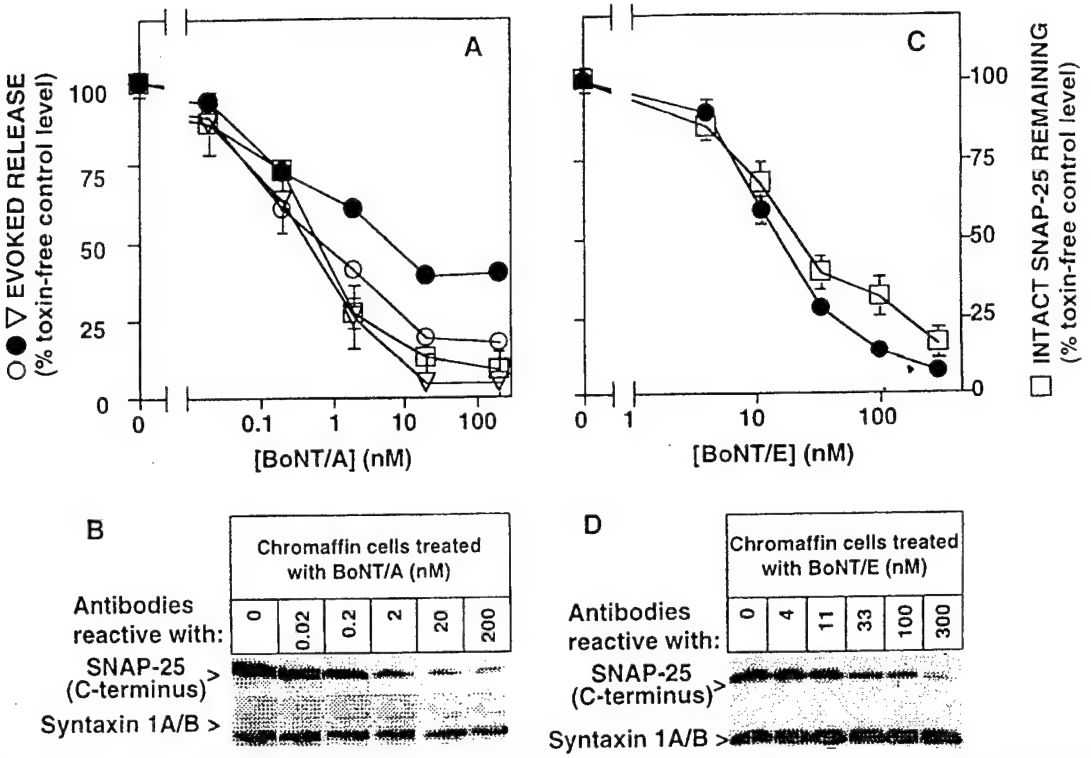


Figure 1. *BoNT/A and E -induced inhibition of secretion from permeabilised chromaffin cells correlates with their proteolyses of SNAP-25.* (A) Intact adreno-chromaffin cells were intoxicated for 24 hr at 37 C with various concentrations of BoNT/A in a low ionic strength buffer before quantifying (± S. D.; n=4) the extents of inhibition of exocytosis over 15 min elicited by 10 μM nicotine (▽) or, following membrane-permeabilisation with 20 μM digitonin, by exposure to 20 μM Ca^{2+} in the absence (○) or presence (●) of 2 mM MgATP (see Lawrence *et al.*, 1996 for details). (B) The amounts of SNAP-25 immunoreactivity remaining (□) in membranes from the same cells was quantified by densitometry (±S. D.; n=3). (C,D) Chromaffin cells were permeabilised by 15 min exposure to 20 μM digitonin in MgATP-containing buffer in the absence or presence of activated BoNT/E before quantifying Ca^{2+}-evoked catecholamine release (●) and SNAP-25 immunoreactivity in the membrane fraction of these cells (□) (see Lawrence *et al.*, 1997). Note that the syntaxin 1A/B content was not altered by either toxin. Error bars encompassed by symbols are omitted. Adapted from Lawrence *et al.*, 1996, 1997.

by Western blotting, that correlated with the extent of inhibition of exocytosis (Fig. 1A). Intact cells proved much less susceptible to intoxication with BoNT/E but Ca^{2+}-triggered secretion was potently inhibited when this toxin was added after digitonin-permeabilisation (Fig 1C), as observed by Bittner *et al.* (1989), with a concomitant cleavage of SNAP-25 (Fig. 1C,D). In contrast, when the BoNT/A-intoxicated cells were permeabilised with digitonin a significant amount of exocytosis could still be elicited by Ca^{2+} (Fig. 1A). Notably, this 'BoNT/A-resistant' release was minimal if MgATP was omitted during the permeabilisation/stimulation step. Such BoNT/A-resistant release, albeit seen only in permeabilised neuroendocrine cells (Bittner *et al.*, 1989; Lomneth *et al.*, 1991), raised the intriguing possibilities that it either occurs independently of SNAP-25 or is mediated by the latter even after its cleavage (Schiavo *et al.*, 1993) by BoNT/A.

Different effects of BoNT/A and /E on Ca^{2+}-evoked secretion from permeabilised chromaffin cells are due to their cleavage of SNAP-25 at separate sites

Overnight exposure of intact chromaffin cells to 66 nM BoNT/A diminished the SNAP-25 C-terminus immuno-reactivity of their membranes to 3.6±1.7 % (n=2) of the level found in neurotoxin-free control cells (Fig. 2A). No changes were detected in the labelling observed with several other antibodies that recognise BoNT/A-insensitive proteins (synaptotagmin, actin, GAP-43, syntaxin and synaptobrevin/cellubrevin). However, there was no reduction in the staining with the monoclonal antibody SMI 81 against SNAP-25 (Fig. 2A) because this reacts with a different epitope located N-terminally to the neurotoxin's cleavage site. Moreover, the electrophoretic mobility of BoNT/A-truncated SNAP-25 fragment ($SNAP\text{-}25_A$) detected by the latter antibody was not

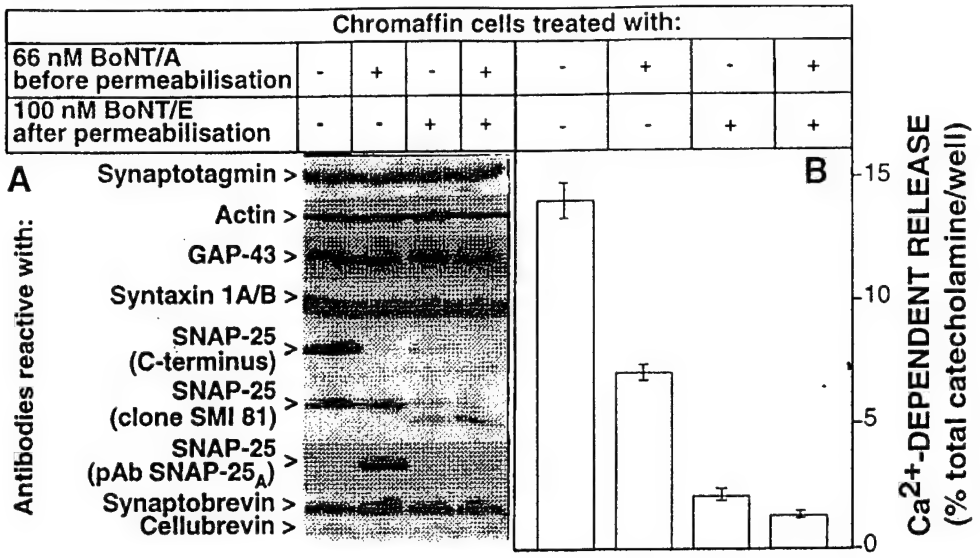


Figure 2. *BoNT/E cleaves BoNT/A-truncated SNAP-25 and blocks BoNT/A-resistant secretion.* Intact chromaffin cells were pre-treated for 24 hr with low ionic strength buffer in absence or presence of 66 nM BoNT/A before permeabilisation for 15 min with 20 μM digitonin in buffer containing MgATP, with and without the inclusion of 100 nM activated BoNT/E. The latter was removed and the cells exposed for 15 min to 20 μM Ca^{2+}; Ca^{2+}-dependent release (B) was calculated (±S.D.; n=4) as previously described (Lawrence et al., 1996). (A) The immunoreactivity signals for SNAP-25 and several other proteins were monitored by Western blotting of the membranous fraction from these cells (Lawrence et al.,1997).

easily distinguished from that for native SNAP-25, confirming that only a small number of C-terminal amino acids had been removed. SNAP-25 cleavage at the established site (Schiavo *et al.*, 1993) was reaffirmed by blotting with an antibody (pAb SNAP-25_A) raised against a peptide designed to mimic the newly-created C-terminus of SNAP-25 (Ekong *et al.*, 1997). Importantly, this antibody was unreactive with SNAP-25 in membranes from neurotoxin-free cells, but gave a strongly labelled band with BoNT/A-poisoned cells (Fig. 2A). When permeabilised and challenged with 20 μM Ca^{2+}, these BoNT/A-poisoned cells gave a secretory response of 50±6 % of that from neurotoxin-free control cells (Fig. 2B), consistent with that noted above (Fig. 1A). Notably, this was abolished by adding BoNT/E during the permeabilisation, an effect attributed to its cleavage of SNAP-25_A as reflected in the production of a smaller fragment (SNAP-25_E) recognised by SMI 81 antibody (Fig. 2A). Moreover, BoNT/E must have cleaved SNAP-25_A at its C-terminus because pAb SNAP-25_A failed to detect the BoNT/E-truncated molecule. As either neurotoxin alone produced near-complete cleavage of SNAP-25 (Fig. 2A), the incomplete inhibition of secretion from permeabilised cells produced by BoNT/A is not simply due to an uncleaved pool of SNAP-25. Accordingly, it has been reported that the BoNT/A-resistant release is not decreased further by re-exposure to BoNT/A after the cells were permeabilised (Lawrence *et al.*, 1996). Although it remains unclear how the nature of the BoNT/A-insensitive secretion differs from the susceptible component, both were blocked by BoNT/E (Fig. 2B) with similar dose-dependencies (Fig. 3). Corroborating evidence implicating SNAP-25_A in the BoNT/A-resistant response was obtained using polyclonal IgG (pAb recS-25) raised against a recombinant form of SNAP-25. Exposure of BoNT/A-treated permeabilised cells to the latter for 30 min before stimulation of exocytosis with Ca^{2+} caused a dose-dependent reduction in the amount of catecholamine release elicited (Fig. 3B); notably, like BoNT/E, pAb recS25 was equally potent in inhibiting secretion from control and BoNT/A-intoxicated cells.

Fusion of primed granules is inhibited by BoNT/E

Previous studies (Holz *et al.*, 1989) have demonstrated that exocytosis from neuroendocrine cells can be dissected into MgATP-requiring and -independent phases. Herein, these distinct components were tested for susceptibility to BoNT/A and /E, added after membrane permeabilisation. It was found that BoNT/E inhibited both the MgATP-requiring and -independent phases of Ca^{2+}-evoked catecholamine release whereas BoNT/A attenuated only the former (Fig. 5A, B). Thus, residues 180-196 of SNAP-25, between the cleavage sites of BoNT/A and BoNT/E,

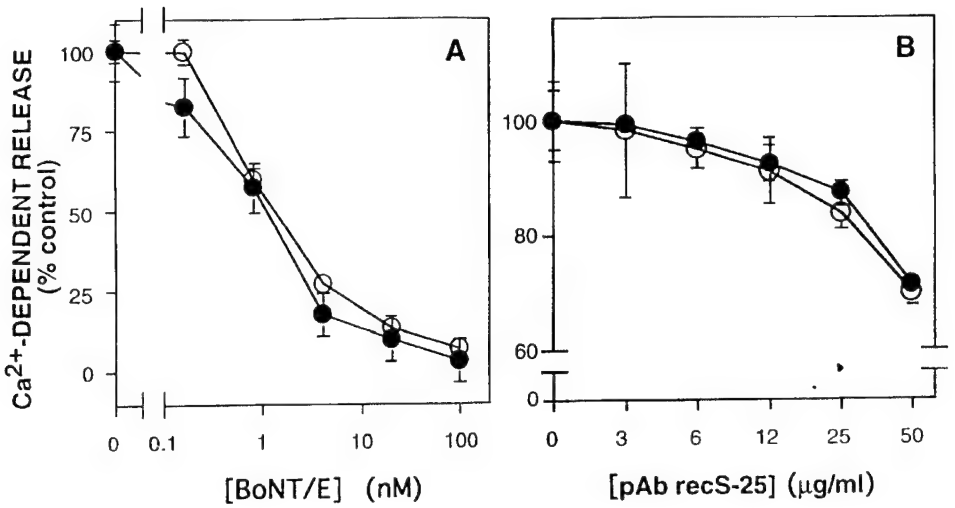


Figure 3. *Dose-dependency for the inhibition of Ca^{2+}-triggered catecholamine release from chromaffin cells by BoNT/E or an antibody to SNAP-25.* Intact control chromaffin cells (●) and those pre-treated with 66 nM BoNT/A (●) were permeabilised by either (A) a 15 min exposure to 20 μM digitonin in MgATP-containing buffer in the absence or presence of of activated BoNT/E, or (B) treatment with the detergent for 30 min in the presence of purified IgG (pAb recS-25) raised against recombinant SNAP-25. In each case, Ca^{2+}-dependent secretion (±S.D.; n=4) was quantified over a subsequent 15 min period; the values were expressed relative to that for the requisite control. IgG isolated from guinea pig pre-immune serum by protein A chromatography did not inhibit release at concentrations up to 50 μg/ml (Lawrence *et al.*, 1997).

seem to be necessary for primed granule fusion unlike those (197-205) removed by BoNT/A (Lawrence *et al.*, 1996).

Discussion

The results presented herein indicate that different regions of SNAP-25 are required at distinguishable stages of the exocytotic process in chromaffin cells. Exposure of intact chromaffin cells to BoNT/A truncated nearly all the SNAP-25 present and abolished the exocytotic responses to nicotinic stimulation (Fig 1A), K^+-depolarisation or exposure to 2 mM Ba^{2+} (Lawrence *et al.*, 1996). Nevertheless, unlike the situation observed with intact cells, when permeabilised in the presence of MgATP and challenged with

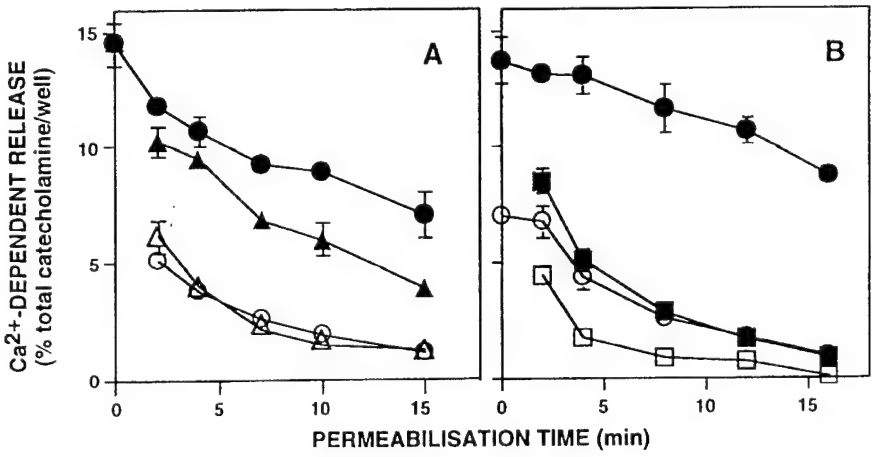


Figure 4. *BoNT/E inhibits both MgATP-requiring and -independent phases of secretion.* Cells were permeabilised in digitonin with (closed symbols) or without MgATP (open symbols), in the absence (●, ○) or presence of 100 nM pre-reduced BoNT/A (A; ▲, △) or /E (B; ■ □) for the times indicated. These solutions were then replaced with neurotoxin-free, digitonin buffer (omitting or including MgATP as during permeabilisation) and Ca^{2+}-dependent release (±S.D.; n=4) after 15 min was determined. Error bars encompassed by symbols are omitted. Adapted from Lawrence *et al.*, 1996, 1997.

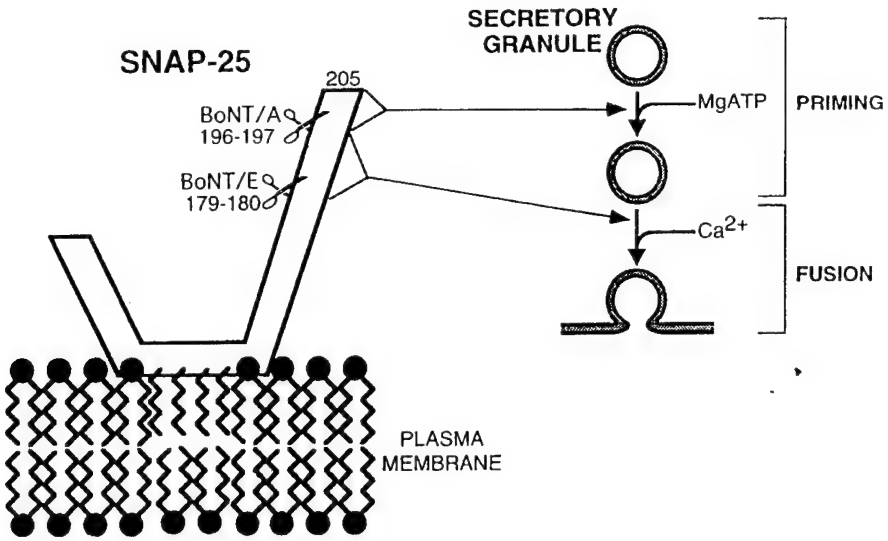


Figure 5. *Blockade by BoNT/A and /E of two distinct steps in exocytosis from chromaffin cells.* The removal by BoNT/A of 9 C-terminal residues of SNAP-25 results in the inhibition of MgATP-dependent priming but does not perturb the Ca^{2+}-stimulated fusion of primed granules. Although priming is abolished after BoNT/A-treatment of intact chromaffin cells, it partially recovers following digitonin-permeabilisation of their plasma-membrane, as reflected in the MgATP-dependent, Ca^{2+}-evoked release; in contrast, this secretion is eradicated by BoNT/E which removes a further 17 amino-acids from the C-terminus of SNAP-25. Moreover, the latter neurotoxin inhibits significantly the MgATP-independent exocytosis of primed granules (Lawrence *et al.*, 1997).

20 μM Ca^{2+}, these same cells secreted a significant amount of catecholamine. Thus, it became apparent that intact SNAP-25 is not essential for an appreciable component of evoked secretion from digitonin-permeabilised cells, though this can be abolished by tetanus toxin or BoNT/C establishing that syaptobrevin and syntaxin are required (Lawrence *et al.*, 1996; Foran *et al.*, 1996). There have been reports that $SNAP\text{-}25_A$, like the full-length protein, can participate with syntaxin and synaptobrevin in the formation of a complex whose disassembly is facilitated by N-ethylmaleimide-sensitive factor (NSF) and soluble NSF-attachment protein (a-SNAP) *in vitro* (Hayashi *et al.*, 1994; 1995; Pellegrini *et al.*, 1995) and in BoNT/A-poisoned synaptosomes (Otto *et al.*, 1995). These raised the possibility of $SNAP\text{-}25_A$ retaining some functionality. This postulation is substantiated herein by demonstrating that the BoNT/A-resistant secretion from permeabilised cells can be abolished by BoNT/E. The latter neurotoxin specifically proteolyses SNAP-25 at residues 179-180 (Schiavo *et al.*, 1993); thus, it removes 17 C-terminal residues from $SNAP\text{-}25_A$. Also, BoNT/E alone prevented secretion from control permeabilised cells; notably, the dose-dependencies for its inhibition of the latter and SNAP-25 proteolysis were essentially identical to that obtained for its blockade of secretion from BoNT/A-pre-treated cells. Thus, intact- and BoNT/A-truncated SNAP-25 appear to be equally susceptible to BoNT/E. The importance of $SNAP\text{-}25_A$ for BoNT/A-resistant release is corroborated by the inhibition obtained with pAb recS25.

The distinguishable effects of BoNT/A and /E reveal some clues to the functional importance of two adjacent C-terminal regions of SNAP-25 in the exocytotic process; a scheme illustrating a likely interpretation of the data is shown in Fig. 5. Residues 180-196, but not 197-205, of SNAP-25 are essential for a significant component of Ca^{2+}-evoked secretion from digitonin-permeabilised chromaffin cells, but the latter region is clearly vital for all of the secretory response from intact cells. Importantly, it was essential to include 2 mM MgATP during the permeabilisation to reveal a significant BoNT/A-resistant response to Ca^{2+}. This means that there were far fewer primed granules, at the time of permeabilisation, in the BoNT/A-poisoned compared to control cells [because, by definition, primed granules can be triggered to fuse by Ca^{2+} alone (see Holz *et al.*, 1989)]. Thus, it can be deduced that residues 197-205 of SNAP-25 are indispensable for the preparation and/or stability of primed granules in intact chromaffin cells (Fig. 5). Such a proposal is supported by the report that $SNAP\text{-}25_A$ can interact in ternary complexes with syntaxin and synaptobrevin *in vitro* as readily as intact SNAP-25, but those generated with the native form display much greater resistance to disassociation in SDS (Hayashi *et al.*, 1994). $SNAP\text{-}25_E$ forms a ternary complex too but this is even less stable, being completely dissociated in SDS.

It has previously been shown (Gutiérrez *et al.*, 1995) that MgATP-independent release persists transiently following chromaffin cell permeabilisation with digitonin; even though such secretion is virtually abolished if cells are exposed overnight to BoNT/A before the detergent treatment, this cannot be inhibited by adding the neurotoxin after the creation of pores in the cell membrane. In contrast, BoNT/E added to permeabilised cells rapidly blocks exocytosis regardless of the presence of MgATP, suggesting that the failure of BoNT/A to attenuate the energy-independent, post-priming phase is not due to inaccessibility of SNAP-25 to the neurotoxin and implicates SNAP-25 residues 180-196 but not 197-205 in this late stage of the secretory process (Fig. 5). A similar conclusion was reached in a recent paper (Banarjee *et al.*, 1996) in which it was demonstrated that BoNT/E prevents the exocytosis of ATP-primed granules from PC 12 cells whereas BoNT/A gives only a partial inhibition.

It remains unexplained why it is only after membrane-permeabilisation that the secretory machinery of chromaffin cells becomes partially resistant to BoNT/A-poisoning; leakage of proteins and/or small molecules from the cell cytoplasm and/or digitonin-induced changes in the structure of the plasma membrane could all be factors which allow $SNAP\text{-}25_A$ to regain functionality after cell permeabilisation.

Acknowledgements

This work was supported by the United States Army Research and Development Command (grant no. DAMD 17-91-Z-1035) and The Speywood Laboratory Ltd. The authors are grateful to Dr C. C. Shone and B. R. DasGupta for providing BoNT/A and /E, and Drs. T.A.N. Ekong and D. Sesardic for the generous gift of an antibody which selectively recognises the C-terminus of BoNT/A-truncated SNAP-25.

References

Ashton AC and Dolly J (1988) *J. Neurochem. 50*, 1808-1816.
Banerjee A, Kowalchyk JA, Das Gupta B.R. and Martin TFJ (1996) *J. Biol. Chem. 271*, 20227–20230.
Bittner, MA, DasGupta, BR and Holz, RW (1989) *J. Biol. Chem. 264*, 10354–10360
Dolly JO, de Paiva A, Foran P, Lawrence G, Daniels-Holgate PU and Ashton AC (1994) *Seminars in Neurosci. 6*, 149–158.
Ekong TAN, Feavers IM and Sesardic, D (1997) *Microbiology (In press)*.
Foran P, Lawrence G and Dolly JO (1995) *Biochemistry 34*, 5494–5503.
Foran P, Lawrence G, Shone CC, Foster K and Dolly JO (1996) *Biochemistry 35*, 2630–2636.
Gutiérrez LM, Canaves JM, Ferrer-Montiel AV, Reig JA, Montal M and Viniegra S (1995) *FEBS Letts 372*, 39–43.
Hayashi T, McMahon H, Yamasaki S, Binz T, Hata Y, Sudhof TC and Niemann H (1994) *EMBO J. 13*, 5051–5061.
Hayashi T, Yamasaki S, Nauenburg S, Binz T and Niemann H (1995) *EMBO J. 14*, 2317–2325.
Hodel A, Schafer T, Gerosa D and Burger MM (1994) *J. Biol. Chem. 269*, 8623–8626.
Holz RW, Bittner MA, Peppers SC, Senter RA and Eberhard DA (1989) *J. Biol. Chem. 264*, 5412–5419.
Lawrence GW, Foran P and Dolly JO (1996) *Eur. J. Biochem. 236*, 877–886.
Lomneth R, Martin TFJ and DasGupta BR (1991). *J. Neurochem. 57*, 1413–1421.
McInnes C and Dolly JO (1990) *FEBS Lett. 261*, 323–326.
McMahon HT, Foran P, Dolly JO, Verhage M, Wiegant VM and Nicholls DG (1992) *J. Biol. Chem. 267*, 21338–21343.
Montecucco C and Schiavo G (1995) *Quarterly Review of Biophysics 28*, 423–472.
Otto H, Hanson PJ, Chapman ER, Blasi J and Jahn R (1995) *Biochem. Biophys. Res. Comm. 212*, 945–952.
Pellegrini LL, O'Connor V, Lottspeich LL and Betz H (1995) *EMBO J. 14*, 4705–4713.
Roth D and Burgoyne RD (1994) *FEBS Lett. 351*, 207–210.
Schiavo G, Santucci A, DasGupta BR, Mehta PP, Jontes J, Benfenati F, Wilson MC and Montecucco C (1993) *FEBS Lett. 335*, 99–103.

Clostridial neurotoxins and membrane traffic at the synaptic terminal

Elaine A. Neale, Linda M. Bowers, and Lura C. Williamson

Laboratory of Developmental Neurobiology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland, USA

Mouse spinal cord neurons in cell culture are an advantageous system for the application of the clostridial neurotoxins (CNTs) to morpho-physiologic analyses in intact, functioning neurons. Large numbers of reproducible preparations are used to compare the effects of individual toxins and to correlate results obtained by diverse methods of assay. These cultures are used to study tetanus and the botulinum neurotoxins by electrophysiology, neurochemistry, immunohistochemistry, and electron microscopy in an effort to provide an integrated concept of toxin interference with neuron function. A complete understanding of the cellular biology of toxin action promises to elucidate generalized mechanisms of membrane trafficking important for neuronal development, survival, and protein targeting.

Cultures

Preparation of the cultures was described first by Ransom *et al.*, (1977) with later modifications detailed by Fitzgerald (1989). Briefly, spinal cords with attached dorsal root ganglia are removed from foetal mice at 12.5 days gestation, minced and trypsin-dissociated. The cell suspension is plated onto collagen (Vitrogen 100)-coated 35 mm culture dishes at moderately high density (10^6 cells/dish) for biochemical studies or, alternatively, onto a preformed feeder layer of cortical glia at lower density (2-3 x 10^5 cells/dish) for morphologic analyses. Within a few hours of plating, the neurons begin to extend processes (Figure 1). Synaptic interactions are recorded electrophysiologically two to three days after plating, and the frequency of synaptically connected pairs of neurons plateaus after approximately three weeks (Jackson *et al.*, 1982; Westbrook and Brenneman, 1984). Postsynaptic neurons respond to the stimulation of presynaptic cells with either excitatory or inhibitory postsynaptic potentials (EPSPs or IPSPs). For most of the studies described below, the cultures are at least three weeks old.

Address all correspondence to: Elaine A. Neale, NIH, LDN, IRP, NICHD, 49-Convent Drive, MSC 4480, Bldg. 49, Rm. 5A38, Bethesda, MD 20892-4480 USA
Phone: 301-496-6419 FAX: 301-496-9939
email: eneale@codon.nih.gov

Morphology

A number of morphologic techniques are available for the visualization of synaptic terminals. Figure 2 illustrates the information on cellular anatomy that is provided by the intracellular injection of horseradish peroxidase (HRP) (Neale *et al.*, 1978). Electron microscopy reveals that the neuronal soma and dendrites in these mature, high density cultures are almost totally covered with synaptic terminals, and that the HRP-labelled swellings seen with light microscopy correspond to terminals derived from the injected neuron. This technique, then, allows an analysis of the morphologic substrate underlying the synaptic connection between the studied neuron pair. More recent methodologies are used to visualize all synaptic terminals in a preparation; e.g. immunohistochemistry for synaptic vesicle proteins. Additionally, electrically "active" terminals can be detected by the activity-dependent uptake of HRP (Heuser and Reese, 1973) or of the fluorescent dye, FM1-43 (Betz and Bewick, 1992; Henkel *et al.*, 1996).

Electrophysiology

Statistical analysis of the electrophysiology and morphology of synaptic connections between spinal cord neurons in culture (Neale *et al.*, 1983; Nelson *et al.*, 1983; Pun *et al.*, 1986) shows that for many connections, there are a large number of "silent" synapses. Further, the analysis suggests that, in individual active terminals, an action potential triggers

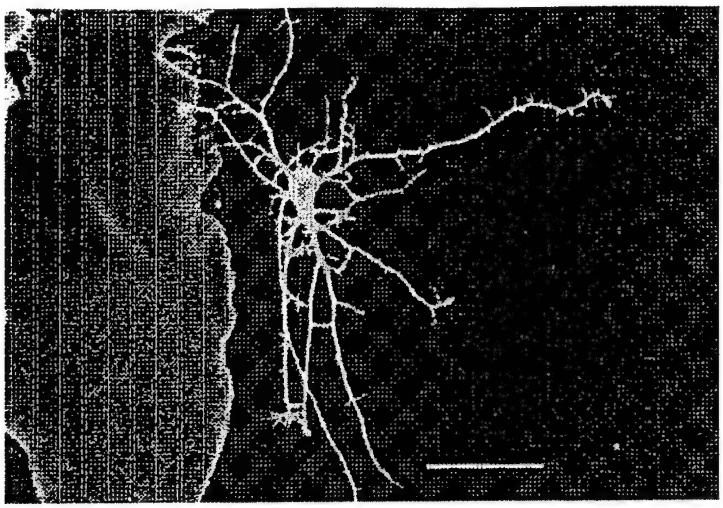
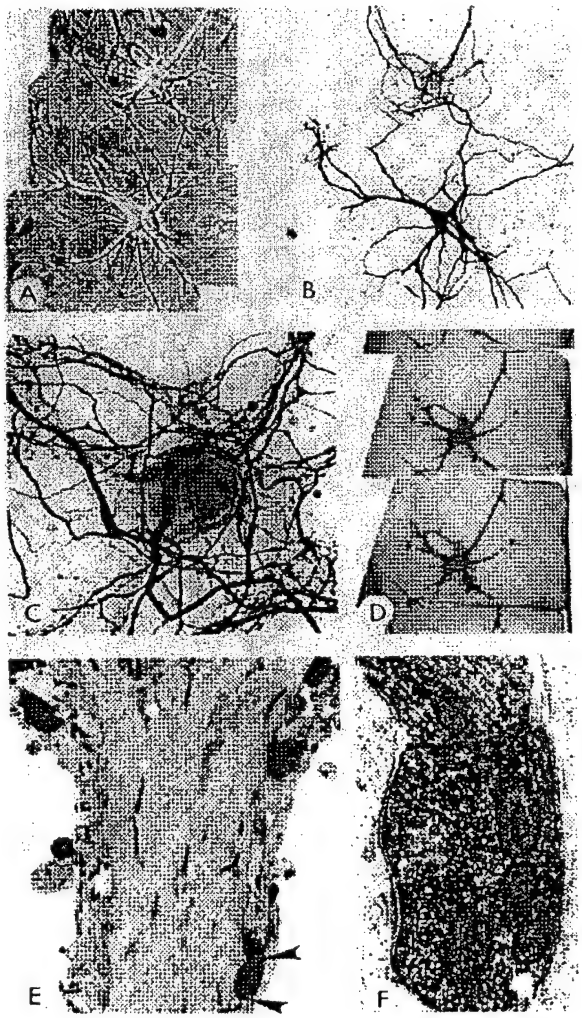


Figure 1. Living spinal cord neuron in dissociated cell culture for 72 hr and stained using Fragment C of tetanus toxin with monoclonal antibody 18.2.12.6 (Neale *et al.*, 1993) and a fluorescent secondary antibody. Neurite development within a few days of plating is considerable. Magnification bar = 50 μm.



the exocytosis of a single synaptic vesicle. Such data raise significant questions for the cellular neurobiologist: are there morphologic features which allow the discrimination of "silent" synapses? What drives the movement of synaptic vesicles to the release site? What directs fusion of vesicles with the specific region of the plasma membrane at the release site, and not with other membranous structures in the terminal? What process links vesicle fusion with the action potential and arrests the machinery after a fusion event? What is the molecular mechanism which retrieves "fused" synaptic vesicle membrane from the terminal plasma membrane and recycles this membrane to maintain a constant supply of vesicles? We believe that the CNTs may be used to probe the

Figure 2. HRP injection of the presynaptic neuron in an excitatory synaptic connection identified by electrophysiology. A) Phase contrast micrograph of a pair of synaptically connected neurons. X100. B) The presynaptic neuron was injected with HRP; its axonal branches course in the direction of the physiologically identified postsynaptic neuron. X95. C) HRP-labelled axonal processes invest the postsynaptic neuron, and labelled varicosities contact its surface. X520. D) The culture was embedded and the area containing the postsynaptic neuron was removed and sectioned for electron microscopy. E) Electron micrograph of a dendrite segment of the postsynaptic neuron. The surfaces of the dendrites and soma are encrusted with axonal processes and boutons. HRP-labelled structures are discriminated easily. Arrowheads mark the bouton shown in F). X5000. F) Synaptic ultrastructure of one of the boutons mediating the analyzed synaptic connection. X36,000. Reprinted with permission from Neale and Nelson (1992).

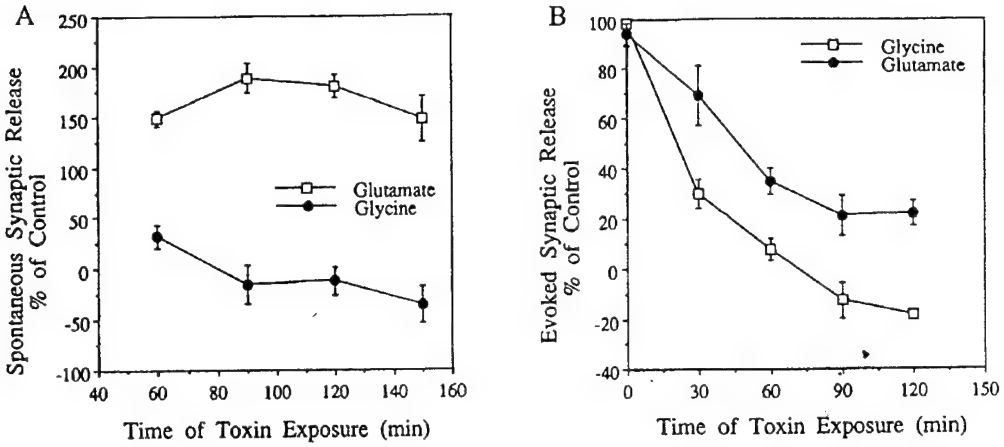


Figure 3. Comparison of tetanus toxin (0.6 nM) effects on spontaneous (A) and evoked (B) synaptic release from 3-week-old spinal cord cultures radiolabelled with [^{3}H]glycine or L-[^{3}H]glutamine. Error bars represent SEM. A) Tetanus toxin exposure causes an increase in spontaneous release of the excitatory neurotransmitter glutamate and a concomitant decrease in release of the inhibitory neurotransmitter glycine. This imbalance correlates with the convulsant activity recorded electrophysiologically and the spastic paralysis seen clinically. n = 8. B) Potassium-evoked release of both neurotransmitters is depressed by tetanus toxin, although the effect on the inhibitory transmitter occurs more rapidly. n = 6. Reprinted with permission from Williamson *et al.*, 1992.

cell biology which governs these membrane movements.

Clostridial Neurotoxins

The physiologic effects of tetanus toxin (TeNT) in spinal cord cell cultures have been examined (Bergey *et al.*, 1983). In synaptically connected pairs of neurons (Bergey *et al.*, 1987), the frequency of IPSPs declines shortly after addition of TeNT to the culture medium whereas the frequency of EPSPs is maintained for several hours. During this interval, action potentials cluster into paroxysmal depolarizing events (PDEs); this convulsant activity thus mimicks the action of TeNT at central synapses *in vivo*. At the reported toxin concentrations, all spontaneous synaptic activity, both inhibitory and excitatory, disappears after several hours and the culture remains electrically quiescent for weeks.

Synaptic activity in these cultures can be monitored also by the biochemical assay of spontaneous or of evoked release of neurotransmitter (Williamson *et al.*, 1992). As shown in Figure 3A, the biochemical assay after TeNT exposure yields results similar to the electrophysiologic measurements; i.e., the *spontaneous* (network-driven) release of inhibitory neurotransmitter (glycine) is depressed shortly after TeNT addition whereas the release of an excitatory transmitter, glutamate, doubles. This interval of enhanced excitation mirrors the PDEs recorded electrophysiologically, and when the toxin concentrations are comparable, the temporal correspondence between the two studies is very similar. Again, all transmitter release ceases after several hours. For most experiments, the release of transmitter *evoked* by potassium depolarization is assayed. The potassium-evoked release of glycine is depressed several hours before that of glutamate (Figure 3B). Interestingly, when sufficiently low concentrations of toxin are used such that the recovery of synaptic function might be predicted (Habig *et al.*, 1986), the release of glutamate is restored before that of glycine (Williamson *et al.*, 1993). Thus, inhibitory neurotransmission appears to be more sensitive to TeNT than excitatory transmission Williamson and Halpern, 1997. Evoked release of neurotransmitter has been assayed also in spinal cord cell cultures after exposure to each of the botulinum neurotoxins (BoNTs). In spite of the fact that BoNTs *in vivo* act at the nerve-muscle synapse to block release of acetylcholine, BoNT D and TeNT show similar potency for blocking glycine release in these central synapses, followed by BoNTs A, B, G, and C, which are about two orders of magnitude less potent (Williamson *et al.*, 1995). In this analysis, BoNTs E and F are ineffective.

Exocytosis

The CNTs have contributed significantly to our understanding of the mechanism of synaptic vesicle exocytosis (Montecucco and Schiavo, 1995 and papers in these proceedings). The demonstration that

the toxins are zinc endopeptidases which act *in vitro* on synaptic proteins homologous to those which function in vesicle docking and fusion in yeast and at the mammalian Golgi apparatus (Ferro-Novick and Jahn, 1994) lends credence to the idea that these proteins are critical for synaptic vesicle fusion and neurotransmitter release. Immunohistochemistry of spinal cord cultures has localized VAMP (vesicle associated membrane protein; cleaved by TeNT and BoNTs B, D, F, and G) to the same sites as the synaptic vesicle membrane proteins, synaptophysin and synapsin. SNAP-25 (cleaved by BoNTs A, E, and C) and syntaxin (cleaved by BoNT C) appear more diffuse along axonal membranes. The spinal cord cultures allow a correlation of the cessation of synaptic function with a toxin's cleavage of its specific protein substrate (Williamson *et al.*, 1996); e.g., when transmitter release is blocked by TeNT, immunohistochemistry of cultures fixed *in situ* demonstrates that VAMP is no longer detectable despite the persistence of staining for synaptophysin or synapsin. Similarly, immunohistochemistry using antibodies against the carboxyl terminus of SNAP-25 fail to detect this portion of the molecule in cultures shown to be blocked with BoNT A. Interestingly, in contrast to *in vitro* experiments (Blasi *et al.*, 1993; Schiavo *et al.*, 1995), BoNT C cleaves not only syntaxin but also has a secondary action on SNAP-25 in intact neurons (Osen-Sand *et al.*, 1996; Williamson *et al.*, 1996) and permeabilized chromaffin cells (Foran *et al.*, 1996).

Electron microscopy of spinal cord cultures fixed after the onset of TeNT-induced electrical quiescence reveals an atypical accumulation of synaptic vesicles at the active zone, consonant with an effect of this toxin on synaptic vesicle fusion (Neale *et al.*, 1989;

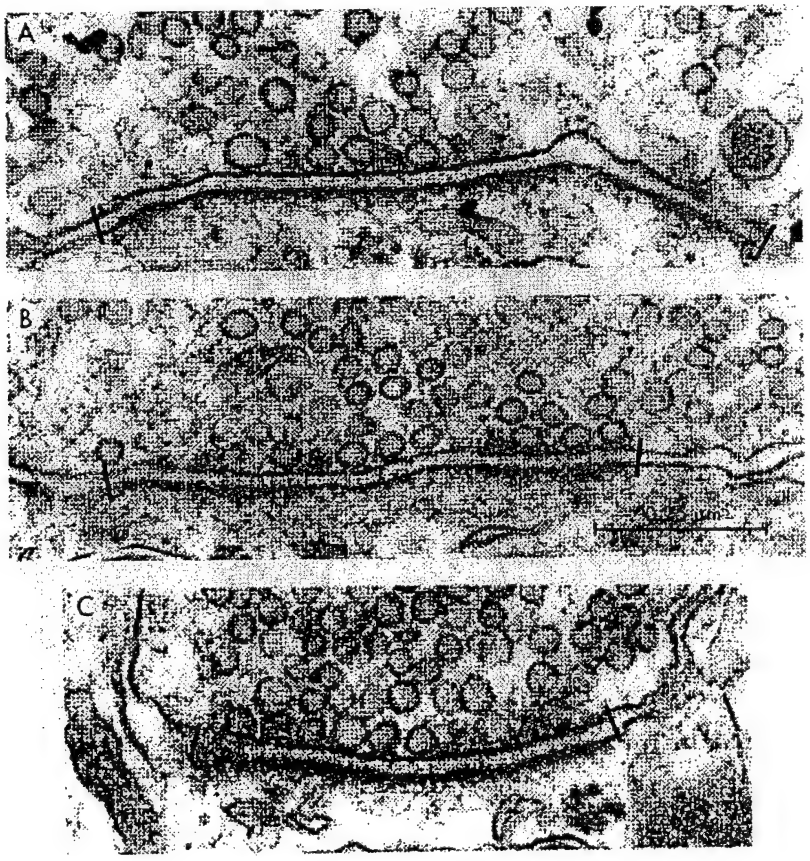


Figure 4. Toxin effects at the synapse. In an untreated preparation (A), a number of synaptic vesicles align at the presynaptic membrane of the release site (marked with vertical bars). This number is reduced in potassium-stimulated cultures (not shown). In tetanus- (B) and botulinum neurotoxin A (C)-exposed cultures, when neurotransmitter release is abolished, approximately twice the number of synaptic vesicles are seen to occupy the release site, suggesting that the block in neurotransmitter release is mediated by an arrest of vesicle fusion.

1993). Counts of vesicles in terminals blocked by TeNT, BoNT A, or BoNT B indicate that approximately twice the number of synaptic vesicles are aligned at the active zone and within 10 nm of the presynaptic membrane in toxin-treated as compared to control cultures (Figure 4). Thus, at a time when transmitter release is totally blocked and when toxin substrates have been proteolyzed, synaptic vesicles appear unable to fuse with the target membrane. This observation fully supports an effect of the CNTs on synaptic vesicle exocytosis.

Endocytosis

As mentioned above, uptake of the styryl dye, FM1-43, is accepted as an indication of synaptic activity (Betz and Bewick, 1992). Dye uptake results from its association with membrane that is endocytosed and recycled into synaptic vesicles as a direct consequence of stimulated exocytosis. In spinal cord cultures, potassium stimulation in the presence of calcium and FM1-43 for five minutes results in considerable fluorescent labeling of synapse-like structures. That the labeled cultures can be de-stained by a second round of stimulation (potassium with calcium in the absence of the dye) is taken as evidence that the dye is associated with recycled synaptic vesicles which have the capacity to be "released" (Henkel *et al.*, 1996). Essentially no staining results from incubation with the dye in the presence of potassium without calcium, or in calcium without potassium. Once loaded, the preparation cannot be destained by potassium without calcium. Thus, stimulation-induced loading attests to the occurrence of the endocytosis component of synaptic vesicle recycling, whereas the consequent destaining argues for the occurrence of vesicle exocytosis.

As expected, it is not possible to demonstrate activity-dependent uptake of FM1-43 in cultures blocked by TeNT or by BoNTs B, C, or D (at 10 ng/ml for 16 hr). In a surprising contrast, FM1-43 uptake persists in cultures exposed to 10-300 ng/ml BoNT A; i.e., cultures in which neurotransmitter release is not detectable, SNAP-25 is cleaved, and synaptic vesicles are accumulated at the active zone. There is no dye uptake in the absence of calcium. Stained preparations cannot be destained, confirming that exocytosis is impaired.

The uptake experiments can be repeated substituting HRP (10 mg/ml for 5 min) for FM1-43 in the stimulation medium, and allowing a 30 min wash interval before fixation for electron microscopy (Figure 5). Horseradish peroxidase labelling of synaptic vesicles in control cultures is evident. As expected, very few vesicles are labelled in TeNT-blocked cultures. However, in cultures in which synaptic release is blocked by BoNT A, there is unequivocal HRP labelling of synaptic vesicles, even at toxin concentrations as high as 200 ng/ml. Labeled vesicles are seen in every vesicle-containing terminal. When similar cultures are fixed immediately after stimulated uptake, coated vesicles containing HRP reaction product are found easily. Therefore, it appears that synaptic vesicle recycling can be stimulated in BoNT A-blocked cultures in which exocytosis had not immediately preceded endocytosis (Neale *et al.*, 1995), and that the recycling involves conventional clathrin-coated structures as described initially at the neuromuscular junction following electrical stimulation (Heuser and Reese, 1973).

There is no evidence for potassium-evoked release of glycine or glutamate in these cultures. However, to strengthen the observation that exocytosis had not occurred, control and BoNT A-blocked cultures are loaded with HRP and stimulated again. Depletion of HRP from synaptic vesicles is comparable to destaining of FM1-43; both constitute evidence for exocytosis. In these experiments, HRP is present at 8 mg/ml and the stimulation interval for loading is two minutes. In control cultures, approximately 10% of synaptic vesicles are labeled with HRP. When the HRP-loading is performed in the absence of calcium, labelled vesicles are rare. In BoNT A-blocked cultures, 6% of synaptic vesicles are labelled. When the control terminals are subjected to a second round of stimulation in the absence of HRP, the percent of labelled vesicles decreases to about 2.5%, providing evidence of exocytosis. When calcium is removed from the stimulation medium, the percentage of labelled vesicles remains at about 11%. In HRP-loaded, BoNT A-blocked terminals, re-stimulation with potassium and calcium does not result in a depletion of HRP; labelled vesicles continue to constitute about 9% of total vesicles. This finding supports the argument that exocytosis had not been triggered by potassium depolarization of BoNT A-exposed cultures.

The source of this recycled membrane is not at all clear. It remains to be demonstrated that the recycled vesicles contain the protein complement of true synaptic vesicles. Whatever the membrane source, it is endocytosed in the face of a block in vesicle exocytosis induced by BoNT A, but not in terminals blocked by TeNT. The data suggest that SNAP-25 is not critical for synaptic vesicle membrane retrieval, but leave open the possibility that VAMP might play a role in synaptic vesicle recycling. BoNT E *in vitro* also cleaves SNAP-25, but in our hands, this serotype is not an effective inhibitor of transmitter release from central synapses in spinal cord cultures. A study of its effects on vesicle recycling will be undertaken using nerve-muscle preparations (see below).

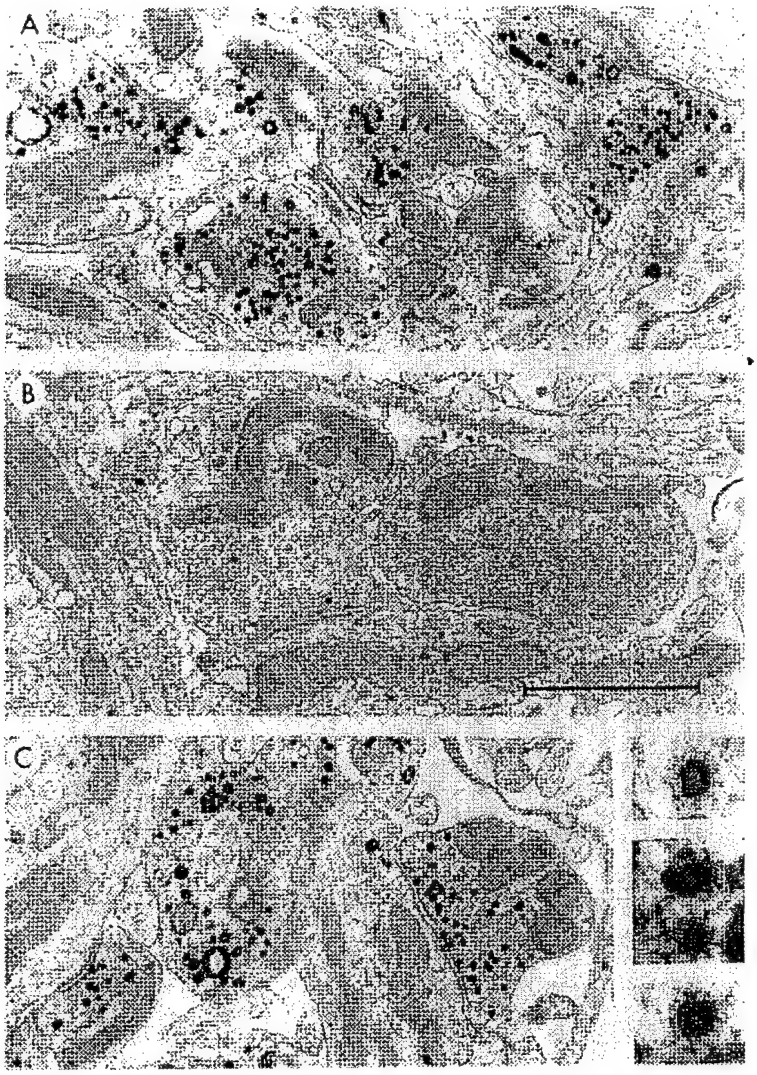


Figure 5. Potassium-stimulated calcium-dependent uptake of HRP. Three-week-old cultures were incubated with 56 mM KCl, 2 mM $CaCl_2$ and 10 mg/ml HRP for 5 min at room temperature. The cultures were rinsed for 30 min and fixed for electron microscopy. In the control preparation (A), a sizeable number of vesicles contain the electron dense reaction product. The labelled vesicles have endocytosed HRP during the course of vesicle recycling. Vesicle membrane retrieval (by endocytosis) follows closely the vesicle exocytosis triggered by potassium stimulation. When neurotransmitter release (vesicle exocytosis) is blocked by tetanus toxin (B), very few vesicles are recycled following potassium stimulation. In contrast, a similar block in neurotransmitter release induced by botulinum neurotoxin A (C) does not prevent vesicle membrane retrieval with potassium stimulation. Insets: Coated vesicles containing HRP-reaction product indicate that the retrieval process in BoNT A-blocked cultures is similar to that described in normal preparations.

Neurotoxicity

Membrane trafficking at the synaptic terminal appears to be altered grossly after exposure to BoNT C. As mentioned above, treatment with BoNT C (10 ng/ml for 16 hr) results in a block in neurotransmitter release, failure to take-up FM1-43 with stimulation, and the proteolysis of SNAP-25 and syntaxin. Additionally, in mature cultures, this toxin leads to swelling of synaptic terminals, the accumulation of extraneous membrane organelles in the terminals, and ultimately terminal degeneration followed by cell death within several days (Kurokawa *et al.*, 1987; Osen-Sand *et al.*, 1996; Williamson and Neale, unpublished observation). The toxic effects are limited to neurons in the cultures, and are not

observed after exposure to any of the other CNT's. BoNT C has similar devastating effects on developing neurons. When freshly dissociated spinal cord neurons are plated in the presence of BoNT C, neuronal development is inhibited severely, and no neurons survive beyond five days of toxin treatment. The mechanism(s) mediating these effects is unknown. However, in short term experiments with newly plated sensory neurons, BoNT C causes growth cone collapse and inhibition of neurite outgrowth, suggesting its interference with membrane trafficking events other than those implicated in synaptic vesicle fusion (Igarashi *et al.*, 1996). Since both BoNTs A and C cleave SNAP-25 (while only BoNT C cleaves syntaxin), a comparison of the effects of these two toxins might discriminate whether SNAP-25 or syntaxin were critical for neurite outgrowth. In young spinal cord cultures, BoNT A has no effect on neuron survival or neurite outgrowth although it clearly acts to cleave SNAP-25. The question remains open, however, since both BoNTs A and C compromise neuron development in cultures prepared from rat cerebral cortex or hippocampus (Osen-Sand et al., 1996).

Future Studies

The spinal cord cultures allow the study of neuronal functions which involve membrane traffic and are impaired by the CNTs. The intriguing and unexplained difference in intracellular routing between TeNT and the BoNTs would be better approached at the neuromuscular junction where BoNT acts effectively and TeNT does not. Multicompartment nerve-muscle co-cultures (Parfitt *et al* 1994) are being used to study the effects of neuronal activity during development on the stabilisation/elimination of synaptic contacts (Nelson *et al.*, 1989; Neale *et al.*, 1991; Liu *et al.*, 1994; Li *et al.*, 1996). These same preparations hold substantial promise for comparisons between TeNT and the BoNTs, with reference to differences in efficacy and to differences in toxin routing. We anticipate that, just as these toxins have moved forward our understanding of synaptic vesicle exocytosis, study of the CNT's will yield further insights into the mechanisms of protein targeting.

Acknowledgements

We are grateful to Ms. Sandra Fitzgerald and to Ms. Veronica Dunlap for preparing the cultures used in our experiments, and to Ms. Sara Behar for her help with morphometric analyses.

References

Bergey GK, Bigalke H and Nelson PG (1987) Differential effects of tetanus toxin on inhibitory and excitatory synaptic transmission in mammalian spinal cord neurons in culture: a presynaptic locus of action for tetanus toxin *J Neurophysiol* **57**, 121–131.

Bergey GK, Macdonald RL, Habig WH, Hardegree MC and Nelson PG (1983) Tetanus toxin: convulsant action on mouse spinal cord neurons in culture *J Neurosci* **3**, 2310–2323.

Betz WJ and Bewick GS (1992) Optical analysis of synaptic vesicle recycling at the frog neuromuscular junction *Science* **255**, 200–203.

Blasi J, Chapman ER, Yamasaki S, Binz T, Niemann H and Jahn R (1993) Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-1/syntaxin *EMBO J* **12**, 4821–4828.

Ferro-Novick S and Jahn R (1994) Vesicle fusion from yeast to man *Nature* **370**, 191–193.

Fitzgerald SC (1989) Dissociated spinal cord-dorsal root ganglion cultures on plastic tissue culture dishes and glass coverslips and wells. In Shahar A, deVellis J, Vernakakis A and Haber B (eds) : *A Dissection and Tissue Culture Manual of the Nervous System.* New York : Alan R. Liss, In.; pp 219–222.

Foran P, Lawrence GW, Shone CC, Foster KA and Dolly JO (1996) Botlulinum neurotoxin C1 cleaves both syntaxin and SNAP-25 in intact and permeabilized chromaffin cells: correlation with its blockade of catecholamine release *Biochem* **35**, 2630–2636.

Habig WH, Bigalke H, Bergey GK, Neale EA, Hardegree MC and Nelson PG (1986) Tetanus toxin in dissosciated spinal cord cultures: long term characterization of form and action. *J Neurochem* **47**, 930–937.

Henkel AW, Lübke J and Betz WJ (1996) FM1-43 dye ultrastructural localization in and release from frog motor nerve terminals *Proc Natl Acad Sci USA* **93**, 1918–1923.

Heuser JE and Reese TS (1973) Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction *J Cell Biol* **57**, 315–344.

Igarashi M, Kozaki S, Terakawa S, Kawano S, Ide C and Komiya Y (1996) Growth cone collapse and inhibition of neurite growth by botulinum neurotoxin C1: a t-SNARE is involved in axonal growth *J Cell Biol* **134**, 205–215.

Jackson MB, Lecar H, Brenneman DE, Fitzgerald S and Nelson PG (1982) Electrical development in spinal cord cell culture *J Neurosci* **2**, 1052–1061.

Kurokawa Y, Oguma K, Yokosawa N, Syuto B, Fukatsu R and Yamashita I (1987) Binding and cytotoxic effects of *Clostridium botulinum* type A, C_1 and E toxins in primary neuron cultures from foetal mouse brains *J Gen Microbiol* **133**, 2647–2657.

Li M, Jia M, Fields RD and Nelson PG (1996) Modulation of calcium currents by electrical activity *J Neurophysiol* **76**, 2595–2607.

Liu Y, Fields RD, Festoff BW and Nelson PG (1994) Proteolytic action of thrombin is required for electrical activity-dependent reduction *Proc Natl Acad Sci USA* **91**, 10300–10304.

Montecucco C and Schiavo G (1995) The structure and function of tetanus and botulinum neurotoxins *Quart Rev Biophys* **28**, 423–472.

Neale EA, Bowers LM, Dunlap V and Williamson LC (1995) Botulinum neurotoxin A blocks synaptic vesicle exocytosis but not membrane retrieval by endocytosis *Mol Biol Cell* **6**, 327a.

Neale EA, Fitzgerald SC, Bowers LM, Yu C, Fields RG and Nelson PG (1991) Multicompartment cell cultures for studies of neuronal interactions. In Conn PM (eds) : *Methods in Neurosciences.* San Diego : Academic Press ; pp 312–349.

Neale EA, Habig WH, Schrier BK, Bergey GK, Bowers LM and Koh J (1989) Applications of tetanus toxin for structure-function studies in neuronal cell cultures. In Nistico G, Bizzini B, Bytchenko B and Triau R (eds) : *Eighth International Conference on Tetanus (Leningrad).* Rome-Milan : Pythagora Press ; pp 58–65.

Neale EA, Macdonald RL and Nelson PG (1978) Intracellular

horseradish peroxidase injection for correlation of light and electron microscopic anatomy with synaptic physiology of cultured mouse spinal cord neurons *Brain Res* **152**, 265–282.

Neale EA and Nelson PG (1992) HRP staining with recording microelectrodes, In Kettenmann H and Grantyn R (eds): *Practical Electrophysiological Methods*. New York: Wiley-Liss; pp 299–303.

Neale EA, Nelson PG, Macdonald RL, Christian CN and Bowers LM (1983) Synaptic interactions between mammalian central neurons in cell culture. III. Morphophysiologic correlates of quantal synaptic transmission *J Neurophysiol* **49**, 1459–1468.

Neale EA, Williamson LC, Bowers LM and Piasecki BA (1993) Synaptic fine structure in tetanus toxin blocked neuronal cell cultures *Mol Biol Cell* **4**, 429a.

Nelson PG, Marshall KD, Pun RYK, Christian CN, Sheriff WH, Jr., Macdonald RL and Neale EA (1983) Synaptic interactions between mammalian central neurons in cell culture. II. Quantal analysis of EPSPs. *J Neurophysiol* **49**, 1442–1458.

Nelson PG, Yu C, Fields RD and Neale EA (1989) Synaptic connections *in vitro*: modulation of number and efficacy by electrical activity *Science* **244**, 585–587.

Osen-Sand A, Staple JK, Naldi E, Schiavo G, Rossetto O, Petitpierre S, Malgaroli A, Montecucco C and Catsicas S (1996) Common and distinct fusion proteins in axonal growth and transmitter release *J Comp Neurol* **367**, 222–234.

Parfitt A, Neale EA, Fitzgerald SC and Nelson PG (1994) Homonymous neuromuscular preparation using the Campenot chamber *Soc Neurosci Abstr* **20**, 1089.

Pun RYK, Neale EA, Guthrie PB and Nelson PG (1986) Active and inactive central synapses in cell culture *J Neurophysiol* **56**, 1242–1256.

Ransom BR, Neale EA, Henkart M, Bullock PN and Nelson PG (1977) The mouse spinal cord in cell culture. I. Morphology and intrinsic neuronal electrophysiologic properties *J Neurophysiol* **40**, 1132–1150.

Schiavo G, Shone CC, Bennett MK, Scheller RH and Montecucco C (1995) Botulinum neurotoxin type C cleaves a single lys-ala bond within the carboxyl-terminal of syntaxins *J Biol Chem* **270**, 10566–10570.

Westbrook GL and Brenneman DE (1984) The development of spontaneous electrical activity in spinal cord cultures. In Caciagli F, Giacobini E and Paoletti R (eds) : *Development Neuroscience: Physiological, Pharmacological and Clinical Aspects*. New York : Elsevier Science Publishers B.V. ; pp 11–17.

Williamson LC, Brown JE and Neale EA (1995) Differential effects of the seven serotypes of botulinum neurotoxin on mammalian central synapses *Soc Neurosci Abstr* **21**, 328.

Williamson LC, Fitzgerald SC and Neale EA (1992) Differential effects of tetanus toxin on inhibitory and excitatory neurotransmitter release from mammalian spinal cord cells in culture *J Neurochem* **59**, 2148–2157.

Williamson LC, Fitzgerald SC and Neale EA (1993) Differential recovery of excitatory and inhibitory neurotransmitter release following tetanus toxin-induced synaptic blockade *Soc Neurosci Abstr* **19**, 1887.

Williamson LC and Halpern JL (1997) Clostridial neurotoxin action in spinal cord neurons after uptake by an alternate pathway. In Tranter HS and Foster KA (ed) : *Biomedical Aspects of Clostridial Neurotoxins*.

Williamson LC, Halpern JL, Montecucco C, Brown JE and Neale EA (1996) Clostridial neurotoxins and substrate proteolysis in intact neurons. Botulinum neurotoxin C acts on synaptosomal-associated protein of 25 kDa *J Biol Chem* **271**, 7694–7699.

Clostridial neurotoxin action in spinal cords after uptake by an alternate pathway

Lura C. Williamson[1] and Jane L. Halpern[2]*

[1]Laboratory of Developmental Neurobiology, NICHD, NIH, and [2]Laboratory of Bacterial Toxins, CBER, FDA, Bethesda, MD 20892

Tetanus toxin (TeNT) and the seven serotypes of botulinum toxin (BoNT) inhibit exocytosis by proteolysis of the SNARE proteins. The vesicle-associated SNARE, VAMP/synaptobrevin, serves as the substrate for TeNT and for four of the seven BoNTs (Montecucco and Schiavo, 1993). In spite of this similar mechanism of action the clinical symptoms seen in cases of tetanus and botulism are very different. These differences can be attributed in part to the peripheral action of BoNTs at the neuromuscular junction versus the central effects of TeNT in the spinal cord inhibitory neurons after retrograde transport. *In vitro* studies also indicate that TeNT and several of the BoNTs vary in potency at inhibitory and excitatory synapses, which may be due to differences in receptor number or distribution. To address this question fusion proteins consisting of the light chains of TeNT or BoNT A linked to a domain from anthrax toxin lethal factor (LF) were prepared. These proteins allow the uptake of TeNT or BoNT A light chain into cells by receptor-mediated endocytosis via the anthrax toxin receptor. The TeNT fusion protein (TeNT-LF), unlike TeNT, inhibited glycine (an inhibitory neurotransmitter) and glutamate (an excitatory neurotransmitter) release equally. These results suggest that the differential potency of TeNT on inhibitory and excitatory neurons may be attributed to receptor distribution rather than light chain activity or substrate availability.

Tetanus toxin (TeNT) and the botulinum neurotoxins (BoNTs) are structurally and functionally closely related molecules that are the etiologic agents of two very distinct diseases (Nieman, 1991). Intoxication with tetanus is characterized by rigidity and spasms in different muscles, especially the muscles of the neck and jaw (trismus). These symptoms result from the blockade of inhibitory neurotransmitter release in the central nervous system. In contrast botulism is manifested by a flaccid paralysis due to the inhibition of acetylcholine release at the neuromuscular junction.

These diverse clinical manifestations of the neurotoxins can be explained, in part, by the differential routing of the toxins in the nervous system. BoNTs remain localized at the neuromuscular junction after binding and internalization. In contrast, the uptake of TeNT at nerve endings is followed by retrograde axonal transport to the spinal cord (Morris *et al.*, 1980). The basis for these distinct trafficking pathways is not well understood.

* Corresponding author: Mailing address; Division of Bacterial Products HFM-437, Food and Drug Administration, Building 29, Room 108, 8800 Rockville Pike, Bethesda, MD 20892
Telephone (301) 434-2424; Fax: (301) 402-2776
e-mail: halpern@ A1.CBER.FDA.GOV

In addition to the distinct effects of TeNT and BoNTs that are seen *in vivo*, *in vitro* studies have also demonstrated that different populations of neurons are differentially sensitive to TeNT and BoNTs. In spinal cord neurons inhibitory neurotransmission is more sensitive to tetanus toxin than excitatory neurotransmission (Bergey *et al.*, 1987, Williamson *et al.*, 1992). In *Aplysia californica*, cholinergic synapses were approximately one hundred-fold more sensitive to BoNT A than to TeNT and this order of potency was reversed at non-cholinergic synapses (Poulain *et al.*, 1990). This difference was eliminated when the toxins were injected intracellularly suggesting that it was due to some aspect of binding and internalization. At the mammalian neuromuscular junction, TeNT and BoNT A blocked neurotransmission in a qualitatively similar manner, however BoNT A was approximately two thousand times more potent (Habermann *et al.*, 1980). The mechanism(s) that underly this disparity are not well understood and could conceivably result from differences at one or more steps during intoxication.

We report here the results of our investigation on the importance of receptors in mediating the sensitivity of neurons to clostridial neurotoxins. Fusion proteins in which the HC of TeNT and BoNT A was replaced with a common receptor binding domain have been

prepared. A combination of immunohistochemistry, immunoblotting and measurements of neurotransmitter release has been used to allow the examination of different aspects of toxin action in spinal cord neurons in culture.

Materials and Methods

Dissociated spinal cord cell cultures from foetal mice were prepared as described (Ransom *et al.*, 1977). Cells were maintained in culture for 3 weeks prior to use in experiments. Neurotransmitter release was measured as described (Williamson *et al.*, 1992) using [^{3}H]glycine and L-[^{3}H]glutamine. Depolarization with 56mM KCl was used to evoke neurotransmitter release.

The fusion protein TeNT-LF was prepared as described (Arora *et al.*, 1994) and the fusion protein BoNT A-LF was prepared using similar methods. The structure of these proteins is shown in Figure 1.

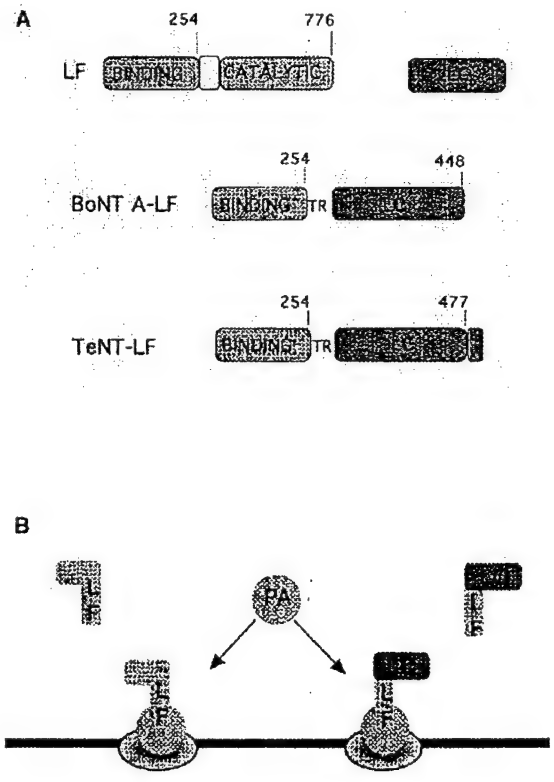


Figure 1 Fusion protein construction and uptake pathway. (A) Fusion proteins corresponding to the amino terminal 254 amino acids from anthrax toxin lethal factor and the light chain from TeNT or BoNT A were prepared using standard molecular biology techniques. (B) The region of lethal factor included in the fusion proteins is sufficient for binding to PA and for delivering heterologous proteins to the cytosol.

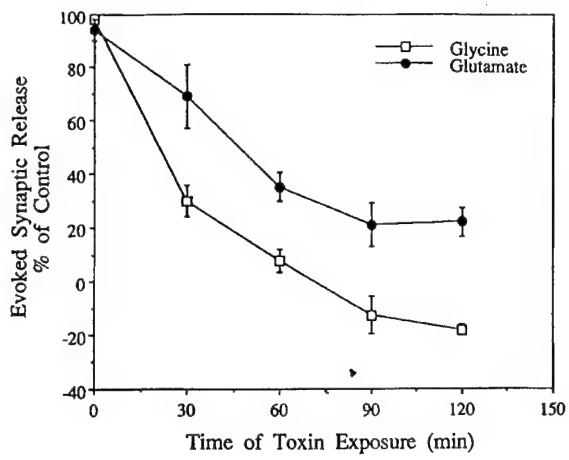


Figure 2 Time course of TeNT action on evoked neurotransmitter release from spinal cord neurons. Cultured neurons were incubated with 0.1 g/ml (0.6 nM) of tetanus toxin for varying periods of time as indicated prior to measuring depolarization-evoked release of glycine and glutamine (Reprinted from *J. Neurochem.* **59**, 2148–2157 (1992).

Results and Discussion

Previous work has demonstrated that in cultured spinal cord neurons inhibitory neurotransmitter release is more sensitive than excitatory neurotransmitter release to intoxication by tetanus toxin (Williamson *et al.*, 1992). A mixed population of spinal cord neurons was incubated with tetanus toxin (0.6 nM) for varying times prior to measuring the evoked release of glutamate and glycine (Figure 2). The results of this experiment demonstrate that the inhibition of glycine release by tetanus toxin occurs more rapidly and to a greater extent than the does the inhibition of glutamate release. Similar studies to quantitate the inhibition of glutamate and glycine release by BoNT A have not been completed yet, however electrophysiological studies (Bigalke *et al.*, 1985) suggested the presence of specific receptors for TeNT and BoNT A in spinal cord neurons.

The inhibition of neurotransmitter release by clostridial neurotoxins can be described as a series of continuous steps that include binding, internalization, translocation to the cytosol, and enzymatic activity. The glycinergic and glutaminergic neurons as shown in Figure 2 may differ in sensitivity to tetanus toxin as a result of a difference in one or more of these steps. For example, the number and/or affinity of receptors for tetanus toxin may vary between neuron types. A second possibility is that the apparent enzymatic activity of the light chain may be greater in one cell type as a result of an activation step. Alternatively, the distribution of the substrate VAMP may

Table 1. Fusion protein activities in cultured spinal cord neurons

Fusion protein	Neurotransmitter release[a]	Enzymatic activity[b]
TeNT-LF	40 ng/ml	Cleavage of VAMP
BoNT A-LF	100 ng/ml	Cleavage of SNAP-25

[a] Concentration of fusion protein at which neurotransmitter release was inhibited to ~ 50% of control levels.
[b] Substrate cleavage in intact cells was measured as described previously (Williamson et al., 1996) and monitored by immunoblot.

differ between cell types, so that one cell type contains
a greater proportion of the total pool in a readily cleavable form.

The role of membrane receptors in determining the sensitivity of different cells to TeNT and BoNT A was assessed by using the fusion proteins described in Figure 1. These proteins comprise the light chain (LC) from either TeNT or BoNT A and the carboxyl terminal 254 amino acids from anthrax toxin lethal factor (LF). These 254 amino acids are necessary for the binding of lethal factor to protective antigen (PA), the receptor binding moiety of anthrax toxin. In the presence of PA both fusion proteins will bind to and be internalized by neuronal cells through an identical receptor rather than through receptors specific for each neurotoxin.

The activities of these fusion proteins are described in Table 1. TeNT-LF and BoNT A-LF inhibit neurotransmitter release in spinal cord neurons and cleave VAMP and SNAP-25, respectively. The activities of the two fusion proteins in spinal cord neurons is dependent on the presence of PA, indicating that uptake is through the anthrax toxin receptor.

Dose-response curves for the inhibition of glutamate and glycine release by TeNT and TeNT-LF are presented in Figure 3. Glycine release was inhibited to a greater extent than glutamate release at concentrations of TeNT between 0.01-1.0 ng/ml. In contrast both neurotransmitters were inhibited equally at all concentrations of TeNT-LF. These data suggest that there are no differences in TeNT light chain activity in inhibitory and excitatory neurons. The increased potency of TeNT in inhibitory neurons therefore is most likely due to a difference in binding or internalization.

The effects of TeNT and TeNT-LF in spinal cord neurons were also compared by measuring cleavage of the substrate VAMP. No difference in the extent of VAMP cleavage by TeNT or TeNT-LF was detected by immunoblot. Surprisingly, it was noted that cleavage of a relatively small percentage of VAMP by TeNT-LF resulted in complete inhibition of secretion (data to be reported elsewhere). In contrast to the

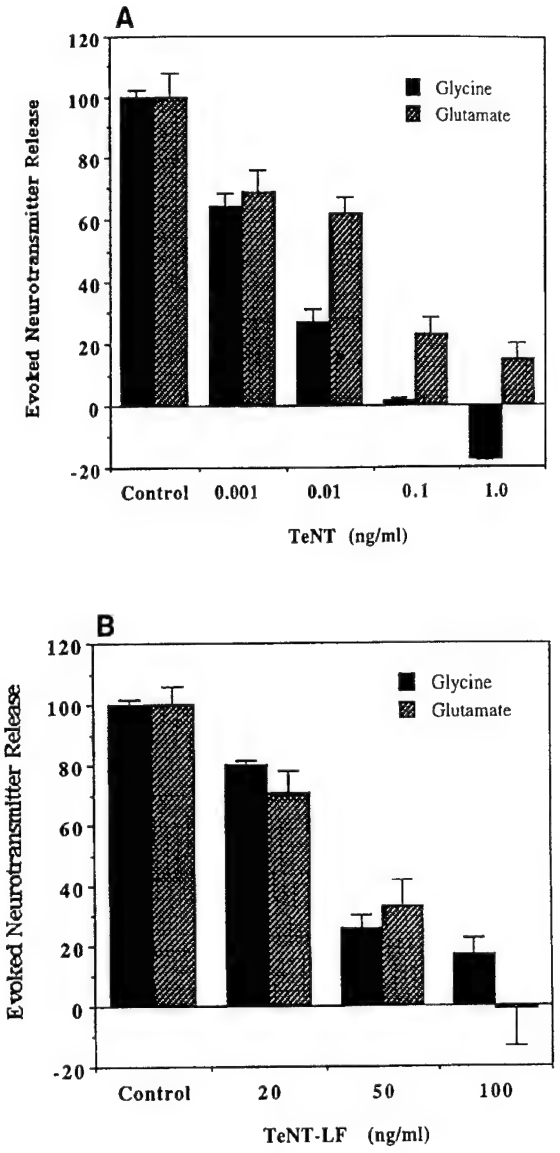


Figure 3 Dose-response of TeNT and TeNT–LF induced inhibition of glycine and glutamate release. Cultured spinal cord neurons were incubated with the indicated concentrations of TeNT (A) or TeNT-LF (B) for 8 h. The cells were labeled with [^{3}H]glycine or [^{3}H]glutamine and depolarization evoked release was measured.

immunoblot results, TeNT and TeNT-LF had different effects on the distribution of VAMP as measured by immunohistochemistry. Treatment with TeNT resulted in synapses with two distinct appearances. In the first group the level of VAMP was comparable to control synapses, but the second group of neurons had little or no immunoreactive VAMP. In contrast, neurons that had been treated with TeNT-LF appeared more uniform, with most neurons having a

reduction in VAMP levels relative to control neurons. These results would appear to be in good agreement with the results in Figure 3. Treatment of a mixed population of spinal cord neurons with TeNT results in preferential uptake by inhibitory neurons and subsequent cleavage of VAMP in these cells which is seen by immunohistochemistry as a loss of VAMP from only certain neurons. In contrast, TeNT-LF is internalized by all neurons which accounts for the more uniform decrease in VAMP that is seen.

The release of both glycine and glutamine was blocked to a similar extent by BoNT A-LF (Data not shown). At 100 ng/ml/BoNT A-LF inhibited release by ~50%. Further experiments with BoNT A and BoNT A-LF should provide useful information regarding receptor distribution on spinal cord neurons.

Specific binding sites for clostridial neurotoxins have been identified using *in vitro* systems such as primary neuronal cultures, established cell lines and partially purified brain membrane fractions. Since these binding sites have not been demonstrated to be functional, the presence of receptors specific for each clostridial neurotoxin has remained speculative. The results reported here suggest that the expression of specific receptors is an important factor in the sensitivity of spinal cord neurons to TeNT and potentially BoNT A. Further elucidation of receptor distribution with our fusion proteins may be useful for characterizing the early steps in clostridial neurotoxin action and in identification of receptor proteins.

References

Arora N, Williamson LC, Leppla SH and Halpern, JL (1994) Cytotoxic effects of a chimeric protein consisting of tetanus toxin light chain and anthrax toxin lethal factor in non-neuronal cells. *J. Biol. Chem.* **269**, 26165–26171.

Bergey GK, Bigalke H and Nelson PG (1987) Differential effects of tetanus toxin on inhibitory and excitatory synaptic transmission in mammalian spinal cord neurons in culture: A presynaptic locus of action of tetanus toxin. *J. Neurophysiol.* **57**, 121–131.

Bigalke H, Dreyer F and Bergey G. (1985) Botulinum A neurotoxin inhibits non-cholinergic synaptic transmission in mouse spinal cord neurons in culture. *Brain Res.* **360**, 318–324.

Habermann E, Dreyer F and Bigalke H (1980) Tetanus toxin blocks the neuromuscular transmission *in vitro* like botulinum A toxin. *Naunyn-Schmiedberg's Arch. Pharmacol.* **311**, 33–40.

Montecucco C and Schiavo G (1993) Tetanus and botulism neurotoxins: a new group of zinc proteases. *Trends Biochem Sci.* **18**, 324–327.

Morris NP, Consiglio E, Kohn LD, Habig WH, Hardegree MC, and Helting TB (1980) Interaction of fragments B and C of tetanus toxin with neural and thyroid membranes and with gangliosides. *J.Biol. Chem.* **255**, 6071–6076

Niemann H (1991) Molecular biology of clostridial neurotoxins. In Alouf JE and Freer JH (eds): *Sourcebook of bacterial protein toxins*. London: Academic Press, pp 303–348.

Poulain B, Mochida S, Wadsworth JDF, Weller U, Habermann E, Dolly, JO and Tauc L (1990) Inhibition of neurotransmitter release by botulinum neurotoxins and tetanus toxin at *Aplysia* synapses: Role of the constituent chains. *J. Physiol.* Paris **84**, 247–261.

Ransom BR, Neale E, Henkart M, Bullock PN and Nelson, PG (1977) The mouse spinal cord in cell culture. *J. Neurophysiol.* **40**, 1132–1150.

Williamson LC, Fitzgerald SC and Neale EA (1992) Differential effects of tetanus toxin on inhibitory and excitatory neurotransmitter release from mammalian spinal cord cells in culture. *J. Neurochem* . **59**, 2148–2157.

Williamson LC, Halpern JL, Montecucco C, Brown JE and Neale EA (1996). Clostridial neurotoxins and substrate proteolysis in intact neurons. Botulinum neurotoxin C acts on synaptosomal-associated protein of 25 kDa. *J. Biol Chem* 271, 7694–7699.

Neurospecific binding of *Clostridium botulinum* type B neurotoxin by recognizing a complex of synaptotagmin and gangliosides

Shunji Kozaki[1*], Yoichi Kamata[1], Tei-ichi Nishiki[2], and Masami Takahashi[2]

[1]Department of Veterinary Science, College of Agriculture, Osaka Prefecture University, Sakai, Osaka 593, and [2]Mitsubishi Kasei Institute of Life Sciences, Machida, Tokyo 194, Japan.

Key words: *C. botulinum*, neurotoxin, synaptotagmin, ganglioside, synaptic vesicle

We previously identified the 58-kDa protein from rat brain synaptosomes, to which *Clostridium botulinum* type B neurotoxin binds only in the presence of gangliosides G_{T1b}/G_{D1a}. This protein was very similar to synaptotagmin, an integral membrane protein of synaptic vesicles. Here, we prepared recombinant synaptotagmins I and II and examined their toxin binding activity. The neurotoxin was found to bind both synaptotagmins incorporated into lipid vesicles in the presence of gangliosides G_{T1b} or G_{D1a}. The dissociation constants for synaptotagmins I and II were comparable to those of low- and high-affinity binding sites respectively in brain synaptosomes. The high-affinity binding of neurotoxin to synaptosomes was inhibited by a monoclonal antibody recognizing the amino-terminal region of synaptotagmin II. A mutant deleting the carboxyl-terminal region of synaptotagmin II still retains the toxin binding activity in the presence of ganglioside G_{T1b} or G_{D1a}. Therefore, the present results indicate that the amino-terminal region of synaptotagmin II is necessary to form the high-affinity binding site by associating with specific gangliosides.

Clostridium botulinum neurotoxin (BoNT) acts on the nerve ending to inhibit neurotransmitter release. All BoNTs (types A to G) are activated by limited proteolysis to yield heavy (~100 kDa) and light (~50 kDa) chains held together by a disulfide bond. The action of BoNT involves binding to receptor via the heavy chain, internalization, translocation through the membrane, and cytosolic action (Simpson, 1986). Recent studies have demonstrated that the light chain is a zinc-dependent protease and specifically cleaves SNAP receptors (SNAREs), synaptobrevin, syntaxin, and SNAP-25 (Montecucco and Schiavo, 1994; Niemann *et al.*, 1994), all of which are critical proteins in synaptic vesicle exocytosis (Rothman, 1994).

Until recently, the nature of the BoNT receptor has not been fully understood, although the binding to the receptor is an obligatory initial step for toxic action. In a series of experiments, we have isolated a 58 kDa protein from rat brain synaptosomes, to which BoNT type B (BoNT/B) binds in the presence of ganglioside G_{T1b} or G_{D1a} (Nishiki *et al.*, 1993; 1994). Partial amino acid sequence and immunological analysis revealed that the 58 kDa protein fraction contained synaptotagmins I and II (Nishiki *et al.*, 1996). Synaptotagmin is a synaptic vesicle membrane protein, highly conserved among a wide range of animal species, which is considered to play a significant role in Ca^{2+}-dependent exocytosis of synaptic vesicles (Littleton and Bellen, 1995). In this report, we examine the characteristics of BoNT/B binding to these synaptotagmins in the presence of specific gangliosides.

*Corresponding author: Shunji Kozaki; Tel: +81-722-52-1161 (ex.2489) Fax: +81-722-52-0341; E-mail: kozaki@center.osakafu-u.ac.jp

Materials and Methods

Materials

BoNT/B was purified and iodinated with $Na^{125}I$ by the chloramine T method (Kozaki, 1979). The toxicity of ^{125}I-BoNT/B was approximately about 80% of that present in unlabelled toxin. Rat brain synaptosomes were prepared as described previously (Nishiki *et al.*, 1994).

Preparation of recombinant synaptotagmin

The cDNAs encoding synaptotagmins I and II were amplified by the polymerase chain reaction with rat

brain cDNA as template (Nishiki *et al.*, 1994). The amplified product was digested with *Nde*I and *Bam*HI and then inserted into the pET3a vector (Novagen). Recombinant synaptotagmins were prepared by solubilization with 20 mM MEGA-9 (nonanoyl-N-methylglucamide) from inclusion bodies. Deletion mutants of synaptotagmin II were constructed into the pMAL-c2 vector (New England Biolabs). The maltose-binding protein (MBP)-fusion proteins were expressed and purified on amylose resin.

Toxin binding assay

The binding activity of recombinant proteins was determined by the acetone precipitation method (Nishiki *et al.*, 1993). The sample was incorporated into phosphatidylcholine vesicles in the presence or absence of gangliosides. The lipid vesicles were incubated for 60 min at 37°C with ^{125}I-BoNT/B. The ^{125}I-BoNT/B bound to the lipid vesicles was separated by filtration through a Millititer-HA plate well. After washing, the radioactivity retained on the filter was determined. Scatchard analysis of the binding data was performed by the computer program SP123 (Ikeda *et al.*, 1991).

Preparation of monoclonal antibodies

A synthetic peptide corresponding to the amino-terminal 20 residues of rat synaptotagmin II (coupled to keyhole limpet hemocyanin) and recombinant synaptotagmin II were used for immunization of BALB/c mice. Monoclonal antibody (mAb) production by hybridoma was examined by ELISA with the synthetic peptide and immunoblotting. A mAb against synaptotagmin I was prepared as described (Nishiki *et al.*, 1994). The mAbs were purified from the ascitic fluid by affinity chromatography on Affi-Gel Protein A.

Results and Discussion

Both of recombinant synaptotagmins I and II exhibited toxin-binding activity only when the ganglioside mixture was incorporated together into lipid vesicles. Neither of the recombinant proteins alone showed any toxin-binding activity. ^{125}I-BoNT/B binding was also observed on the lipid vesicles in the presence of gangliosides G_{T1b} and G_{D1a}, but not G_{M1}, G_{M3}, G_{D3}, or G_{D1b}. ^{125}I-BoNT/B bound to the recombinant proteins in lipid vesicles incorporating a ganglioside mixture in a saturable manner. Scatchard plot analysis revealed a single class of binding site with

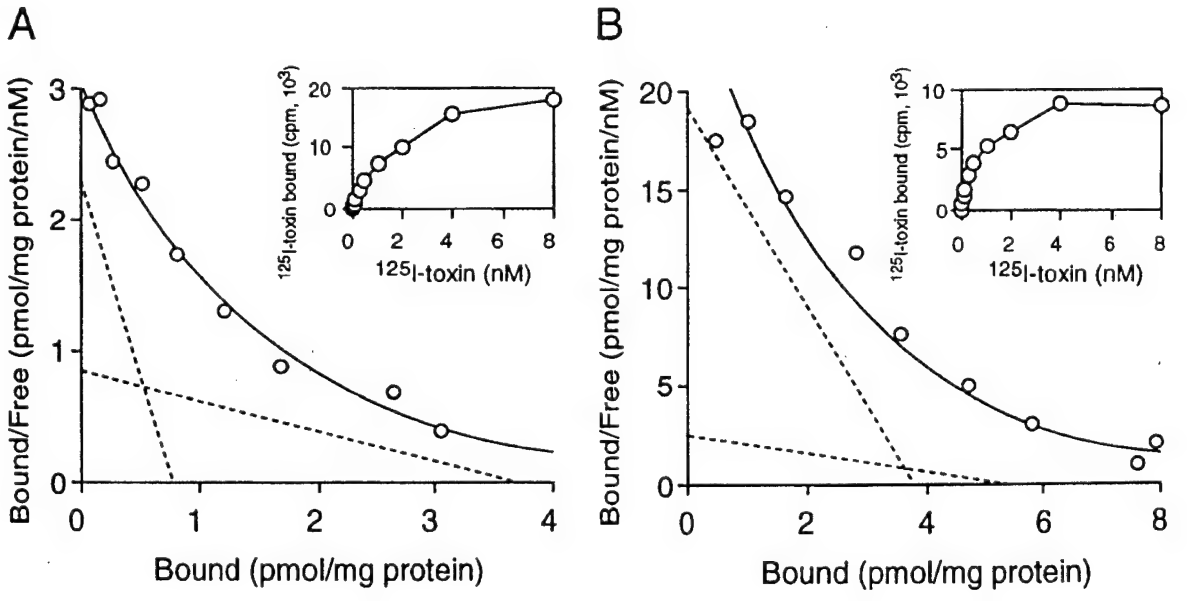


Figure 1. Scatchard plot analyses of ^{125}I-BoNT/B binding to rat brain synaptosomes. Specific binding was determined as the difference between means of triplicate assays in the absence or presence of 200-fold excess unlabeled toxin. A, Scatchard plot and binding saturation curve (inset) for cerebral synaptosomes. B_{max} values for high affinity (Kd = 0.3 nM) and low affinity (Kd = 4.3 nM) sites were 0.79 and 3.71 pmol/mg of protein, respectively. B, Scatchard plot and binding saturation curve (inset) for cerebellar synaptosomes. B_{max} values for high affinity (Kd = 0.2 nM) and low affinity (Kd = 2.1 nM) sites were 3.81 and 5.41 pmol/mg of protein, respectively.

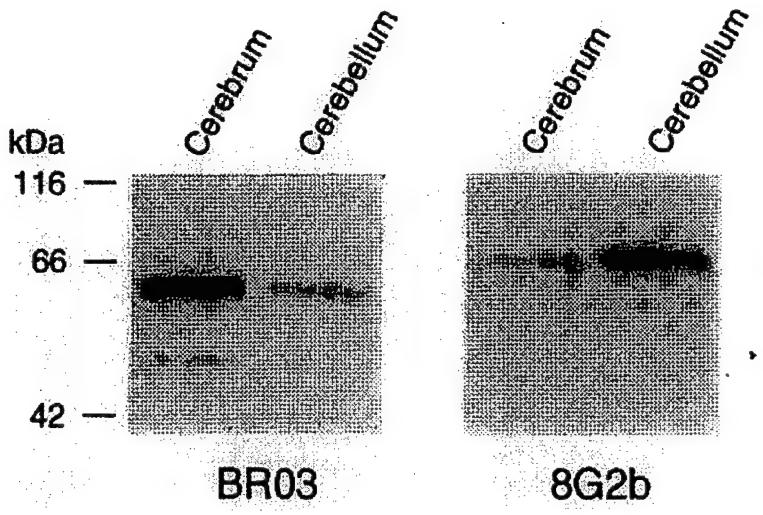


Figure 2. Detection of synaptotagmins I and II in brain synaptosomes. Cerebral and cerebellar synaptosomes were subjected to SDS-PAGE (10 μg/lane, 12.5% gel) followed by immunoblotting probed with monoclonal antibodies against synaptotagmins I (BR03) and II (8G2b). The position of molecular mass standards are shown on the left.

the dissociation constants (Kd) of 2.3 and 0.2 nM for synaptotagmins I and II, respectively.

We examined ^{125}I-BoNT/B binding to cerebral and cerebellar synaptosomes. Scatchard plot analyses of the data showed that these synaptosomes contained two classes of binding site for BoNT/B. The Kds of low- and high-affinity binding sites in both synaptosomes were comparable to those of recombinant synaptotagmins I and II, respectively. The concentration of high-affinity binding site in cerebellar synaptosomes was about 5 times higher than that in cerebral synaptosomes (Fig. 1A and B). Immunoblotting with monoclonal antibodies against synaptotagmins I and II indicated that synaptotagmin II in cerebellar synaptosomes was expressed about 5 times higher than that in cerebral synaptosomes, whereas synaptotagmin I seemed to be involved in cerebrum rather than in cerebellum (Fig. 2). These observations suggest that synaptotagmins I and II form the low- and high-affinity binding sites in brain synaptosomes.

Synaptotagmin has a single transmembrane region and short amino-terminal domain located in the intravesicular space (Perin *et al.*, 1991). However, the amino-terminal domain is exposed at the outside of the nerve terminal after synaptic vesicle exocytosis (Shoji-Kasai *et al.*, 1992), which suggests that the amino-terminal region may constitute BoNT/B binding site. In order to address the question, we firstly examined the inhibitory effect of mAb on BoNT/B binding to synaptosomes. The mAb ST209, recognizing specifically the amino-terminal region of synaptotagmin II, was found to inhibit BoNT/B binding to the high-affinity binding in cerebral synaptosomes. We also conducted the binding experiments with MBP-Stg2N and -Stg2C, encoding amino acids 1-87 and 61-422 of synaptotagmin II, respectively. ^{125}I-BoNT/B showed binding to MBP-Stg2N incorporated into lipid vesicles in the presence of ganglioside G_{T1b} or G_{D1a} (Fig. 3). The Kd for MBP-Stg2N was comparable to that of full length of synaptotagmin II. However, no toxin binding was observed in the lipid vesicles containing MBP-Stg2C

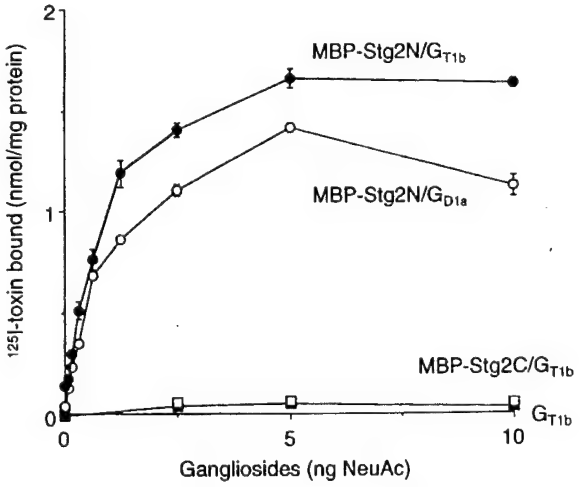


Figure 3. Binding of ^{125}I-BoNT/B to deletion mutant of synaptotagmin II. MBP fusion proteins were incorporated into lipid vesicles in the presence of gangliosides GT1b or GD1a at various concentrations. The data are means ± S.E. of three determinations.

and ganglioside G_{T1b} or ganglioside G_{T1b} alone. These results support the idea that the amino-terminal region in synaptotagmin II is a pivotal portion to form the high affinity binding site for BoNT/B. The C2A domain of synaptotagmins I and II, which is located in the carboxyl-terminal region, bound negatively charged phospholipids in Ca^{2+}-dependent manner (Li *et al.*, 1995). However, the present data clearly demonstrate that only the amino-terminal region of synaptotagmin II alone is sufficient for formation of the BoNT/B binding site, by associating with gangliosides G_{T1b} and G_{D1a}.

References

Ikeda S, Oka J and Nagao T (1991) Effects of four diltiazem stereoisomers on binding of d-cis-[3H]diltiazem and (+)-[3H]PN200-110 to rabbit T-tubule calcium channels. *Eur. J. Pharmacol.* **208**, 199–205.

Kozaki S, (1979) Interaction of botulinum type A, B and E derivative toxins with synaptosomes of rat brain. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **308**, 67–70.

Li C, Ullrich B, Zhang JZ, Anderson RGW, Brose N and Südhof TC (1995) Ca^{2+}-dependent and -independent activities of neural and non-neural synaptotagmins. *Nature* **375**, 594–599.

Littleton JT and Bellen HJ (1995) Synaptotagmin controls and modulates synaptic-vesicle fusion in a Ca^{2+}-dependent manner. *Trends Neurosci.* **18**, 177–183.

Montecucco C and Schiavo G (1994) Mechanism of action of tetanus and botulinum neurotoxins. *Mol. Microbiol.* **13**, 1–8.

Niemann H, Blasi J and Jahn R (1994) Clostridial neurotoxins: new tools for dissecting exocytosis. *Trend. Cell Biol.* **4**, 179–185.

Nishiki T, Kamata Y, Nemoto Y, Omori A, Ito T, Takahashi M and Kozaki S (1994) Identification of protein receptor for *Clostridium botulinum* type B neurotoxin in rat brain synaptosomes. *J. Biol. Chem.* **269**, 10498–10503.

Nishiki T, Ogasawara J, Kamata Y and Kozaki S (1993) Solubilization and characterization of the acceptor for *Clostridium botulinum* type B neurotoxin from rat brain synaptic membranes. *Biochim. Biophys. Acta* **1158**, 333–338.

Nishiki T, Tokuyama Y, Kamata Y, Nemoto Y, Yoshida A, Sato K, Sekiguchi M, Takahashi M and Kozaki S (1996) The high-affinity binding of *Clostridium botulinum* type B neurotoxin to synaptotagmin II associated with gangliosides GT1b/GD1a. *FEBS Lett.* **378**, 253–257.

Perin MS, Brose N, Jahn R and Südhof TC (1991) Domain structure of synaptotagmin (p65). *J. Biol. Chem.* **266**, 623–629.

Rothman J E (1994) Mechanisms of intracellular protein transport. *Nature* **372**, 55–63.

Shoji-Kasai Y, Yoshida A, Sato K, Hoshino T, Ogura A, Kondo S, Fujimoto Y, Kuwahara R, Kato R and Takahashi M (1992) Neurotransmitter release from synaptotagmin-deficient clonal variants of PC12 cells. *Science* **256**, 1820–1823.

Simpson LL (1986) Molecular pharmacology of botulinum toxin and tetanus toxin. *Ann. Rev. Pharmacol. Toxicol.* **26**, 427–453.

Session 7

Detection and Quantitation

The role of WHO standards in the quantitation of biological medicinal products

D H Calam

National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Herts. EN6 3QG.

Keywords: WHO; biological products; bioassays; reference materials; standardization.

The World Health Organisation has a programme that began more than 70 years ago for the preparation and distribution of international standards (reference materials) intended to facilitate the measurement of biological medicinal products on a common worldwide basis. The WHO definition of such products is that they cannot be completely defined in chemical and physical terms alone but require some form of biological measurement to characterise them. The philosophy behind this programme, its scope and operation are described.

Introduction

Why have standards? Formerly, the unit of length differed between countries and even within one country - a situation leading inevitably to confusion when comparisons are necessary. In due course the metre was defined in terms of a metal alloy standard and, in about 1889, copies of this metre were distributed to different governments to serve as the basis for an internationally agreed unit of length.

Similar problems existed in the field of medicines, particularly of biological origin. Variability in quality and potency were known to occur with antitoxins and oxytocin. The discovery of insulin and recognition of its therapeutic potential raised these problems to an international level. It was recognised that to avoid proliferation of units of potency, a single, internationally accepted, stable reference preparation with a unitage defined on the basis of an absolute amount of it was necessary. The experimental methods by which a given substance was measured could differ, and therefore be the subject of development and improvement, but the basis of comparison of a sample with the international reference material would remain the same. Such a reference material would also help reduce variability in quality by providing a firm basis against which to monitor consistency of, and changes and developments in, manufacturing processes. From this starting point, the then League of Nations established an international programme for the preparation and distribution of reference materials for biological products. The first such materials for insulin, oxytocin and vasopressin and arsphenamine were established in 1925, for digitalis in 1926, and for anti-dysentery serum, tetanus antitoxin and ouabain in 1928. Their activities, in International Units (IU), were defined in terms of the weight of a certain quantity of the standard. From this beginning, the programme now administered by the World Health Organization (WHO) has established more than 350 biological reference materials.

The biological substances covered by this programme were and are defined as those used in the prophylaxis, therapy or diagnosis of human and some animal diseases, the potency of which cannot be expressed directly in terms of chemical and physical quantities. From the viewpoint of a specification for a therapeutic product, this means that if, after applying whatever chemical and physical tests are appropriate, an *in vitro* or *in vivo* biological assay is needed to fully define the product, it is a 'biological'. Generally, the less well-defined the structure and composition of the product and the greater its inhomogeneity, the more likely it is to require bioassay. At various times substances for which biological standards have existed include organic arsenicals, steroids, vitamins, antibiotics, peptide and protein hormones, cytokines and growth factors, blood products, vaccines and antitoxins.

The WHO definition makes no statement about the nature of a biological product as regards its structure or origin, nor are chemical and physical tests on it ruled out. As scientific and technical

advances have taken place over the past 75 years, the ability to isolate increasingly complex molecules in high purity and to define them in chemical and physical terms has increased. A number of substances formerly measured by biological assay are now recognised as suitable for control by chemical and physical methods alone, for example steroids, vitamins and some antibiotics. The biological standards for these substances have been discontinued although in a few instances a notional equivalence between IU and weight of the substance have been stated. As methodology continues to improve, it is generally agreed that peptides and small proteins can be adequately defined today without recourse to bioassay on a routine basis.

Establishment of WHO standards

The WHO programme for biological standards is administered from Geneva by a secretariat and the Expert Committee on Biological Standardization (ECBS). The first task of the Committee is to select substances for which WHO biological standards are required. There are currently four designated WHO International Laboratories for Biological Standards, of which the National Institute for Biological Standards and Control (NIBSC) is one. One of these laboratories is designated to obtain candidate materials for assessment, prepare a batch of one or more of these, ensure that the chosen batch is uniform and stable, organise an international collaborative study, analyse the resulting data and report with recommendations to the ECBS. The decision to establish a WHO standard is taken by the ECBS (WHO, 1990). The major criteria considered are set out below.

Uniformity

Many WHO standards consist of lyophilised powders, portions of which can be weighed out. Since their content is expressed on the basis of IU/mg, the potency of a solution of the preparation is readily calculated. However, because many of the standards are hygroscopic, weighing is subject to error. The potency of most standards established now is defined on a 'per ampoule' basis and uniformity of content from ampoule to ampoule is clearly important. In general processing as a liquid gives better uniformity than weighing out a solid. The recommended maximum coefficient of variation for the contents of WHO standards filled in liquid form and before further processing is 0.25% (WHO, 1990), although a wider variation may be justified depending on the material and its intended use. This can be achieved regularly with suitable filling equipment. The reproducibility of fill is monitored by weighing the contents of ampoules taken at regular intervals (for example every 40th ampoule) throughout the filling process.

Stability

While a liquid fill gives better uniformity of ampoule content, stability is favoured by having the standard as a solid in sealed glass ampoules (not capped vials), under an inert gas. For this reason the recommended process is to fill aliquots of a filtered solution of the preparation, often also containing an inert bulking agent, under aseptic conditions into glass ampoules, lyophilise the contents, carry out a secondary desiccation, purge the ampoules with pure, dry nitrogen and seal them. Most WHO standards are stored at –20°C but baseline samples are held at lower temperatures to permit assay comparison should any query arise about stability at a later date.

To avoid the waste of resources in performing collaborative studies on unsuitable material, accelerated degradation studies are carried out. These studies are performed to a design in which ampoules stored at different temperatures are analysed after different storage times relative to duplicate ampoules taken from the main batch at the intended storage temperature (Moss *et al.*, 1994). Suitable assay designs have been developed to permit statistical analysis of the results and prediction of long-term stability (Kirkwood 1977, Tydeman and Kirkwood,1984). All ampoules are coded to prevent operator bias in the analysis. Duplicate baseline samples permit an assessment of assay variation. Extensive evidence of the value of this procedure is summarised for a series of enzyme preparations in Moss *et al.* (1994).

Reliance on accelerated degradation studies is founded on assumptions about validity of the Arrhenius equation and its application to lyophilised biological standards. The equation is valid for a single chemical reaction and postulates a linear relationship between the logarithm of the rate constant of this reaction and the reciprocal of the absolute temperature. The rate constant is derived from the initial (baseline) activity, storage time and activity measured. It is also assumed that the logarithm of potency is related linearly to the storage time at all temperatures. Comparisons reported by Moss *et al* (1994) between predicted and real-time degradation rates confirm the reliability of predictions made based on these assumptions. It is also a general observation that predictions after short storage times tend to overestimate long-term degradation rates and that short-term data deviate more often from the model. These deviations are probably explained by the presence of traces of moisture and oxygen in the sealed ampoules which can give rise initially to other degradation pathways.

The life of a WHO biological standard is normally not less than ten years.

Collaborative study

Once adequate stability is predicted for a candidate preparation, an international collaborative study is organised. This involves expert laboratories of different kinds (regulatory, academic, industrial, clinical) drawn so far as possible from a wide geographical spread. The laboratories are usually encouraged to apply whatever assay procedure they customarily employ. Sometimes the study will involve analysis of several candidate preparations, as in the case of the study currently in progress on botulinum toxin. One outcome is to establish whether apparently similar materials exhibit the same behaviour in different assay systems. The results of the study are subjected to detailed statistical analysis by a biometrician. One preparation is selected as the proposed standard and the report on the study and recommendations are sent to all participants for comment and agreement. The report is then submitted to the ECBS and, if accepted, the particular preparation is established as the WHO biological standard with a declared potency.

WHO standards are made available to national regulatory and control authorities without charge and to others (manufacturers, clinical, academic and research laboratories) on payment of a nominal handling charge. Their use and value can be judged by the distribution of approximately 11,000 ampoules in 1994-5 (WHO, 1996) to all WHO regions. In 1995-6 NIBSC issued more than 62,000 ampoules of all types of biological reference materials including established and candidate WHO materials, regional and national materials and materials filled under contract. This total has increased by 17,000 over a four year period.

WHO standards and medicines control

In many countries, the provision of medicines is under regulatory control. This is exerted by approval (licensing) for clinical trial and for full marketing, by inspection of manufacturers and distributors, and by publication of specifications for established products in pharmacopoeias. The approval process applies to individual products. For a biological for which no WHO standard is available, the manufacturer's reference material and assignment of unitage will usually be accepted by an individual authority, who will also require to be supplied with some of the material for control purposes. However, the manufacturer, a national authority or WHO itself through ECBS may put in motion the process for establishment of a WHO standard. After the establishment of a WHO standard, the unitage will be expressed in International Units but every effort will be made to ensure equivalence between the unit used previously and the IU.

This procedure becomes more complex if more than one apparently equivalent product is approved and no WHO standard exists. A real possibility exists of introduction of non-equivalent units with all the potential and actual confusion that may result. The basis for assignment of potency may differ and the dosages of the products may not be the same. This is at present the case for botulinum toxin. However, candidate materials have been prepared and are undergoing collaborative assessment. A basic principle of biological standardization is that like has to be assayed against like: the test sample and reference material should exhibit parallel response in an assay system. Part of the purpose of the collaborative study using different preparations and different assays is to establish whether preparations of different origin are sufficiently similar for this principle to hold. If not, more than one standard, each with an arbitrary assignment of unitage may be necessary.

When a regional (for example European) or national pharmacopoeial monograph is prepared it will set legally enforceable criteria and test procedures including an assay defining the content of a chemical drug or potency of a biological substance. The biological reference preparations of the European Pharmacopoeia are calibrated against WHO standards and their potency is expressed in IU. Thus the control and testing of products that are the subject of such monographs is traceable directly to the WHO standard and IU.

Limited role of a standard

The availability and use of a WHO standard may lead to improvements in the quality, consistency and comparability of products containing a particular active substance. However, it alone cannot solve all the problems associated with use of medicines for the prevention and treatment of disease. It may be difficult or impossible to devise an assay that is relevant to the clinical application of a product. The individual responses of patients may be so different that the dose has to be titrated individually to ensure that the desired effect is obtained. A standard cannot overcome these problems. However, it can overcome variability of product and sourcing and provide a consistent and secure base against which the other problems can be addressed.

References

Kirkwood TBL (1977) Predicting the stability of biological standards and products. *Biometrics* 33, 736-742.

Moss DW, Maire I, Calam DH, Gaines Das RE, Lessinger JM, Gella FJ and Férard G (1994) Reference materials in clinical enzymology: preparation, requirements and practical interests. *Ann Biol Clin* **52**, 189-198.

Tydeman MS and Kirkwood TBL (1984) Design and analysis of accelerated degradation tests for the stability of biological standards. *J Biol Stand* **12**, 195-224.

WHO (1990) Guidelines for the preparation, characterization and establishment of international and other standards and reference reagents for biological substances. *WHO* Technical Report Series **800**, 181-214.

WHO (1996) WHO Expert Committee on Biological Standardization. Forty-sixth report. *WHO Technical Report Series* (in press).

Definition of units: clinical experience

Geoffrey L. Sheean

The National Hospital for Neurology and Neurosurgery, Queen Square, London, WC1N 3BG, UK

The purpose of this paper is to provide a clinical perspective on the issues of quantitation of botulinum toxin effect and of bioequivalence of different formulations of botulinum toxin (BTX).

Botulinum toxin has revolutionised treatment of dystonia and other conditions characterised by excessive muscular contraction. It has proved to be safe and effective and the list of applications continues to grow. There are only two commercial preparations of botulinum toxin available, Dysport and Botox, both BTX type A. The method by which potency is determined for each is the LD_{50} mouse assay. However, it has been long recognised that these two preparations have quite different potencies; Dysport appeared to be clinically less potent than Botox (Quinn and Hallet, 1989; Brin and Blitzer, 1994). The introduction of other BTX serotypes (e.g. types B and F) into clinical trials showed that they too were not clinically bioequivalent, further emphasising that the LD_{50} mouse assay was not a reliable guide to clinical potency.

In clinical terms, the lack of bioequivalence has some important implications. When changing to another formulation or type of BTX, there is the risk of overtreatment (excessive paralysis and side-effects) or of undertreatment (insufficient weakness), depending upon the direction of change. The former could be fatal or disabling and the latter is disappointing for the patient, wastes time, money and toxin, and gives the patient a potentially immunogenic exposure without the benefits of a clinical response. A side issue is that the lack of bioequivalence must pose problems for pharmaco-economical studies in evaluating the cost-effectiveness of formulations or types of toxins.

Address for Correspondence: Dr. Geoffrey L Sheean, Department of Clinical Neurophysiology, The National Hospital for Neurology and Neurosurgery, Queen Square, London, WC1N 3BG United Kingdom. Tel: (+44) 171 829 8752; Fax: (+44) 171 713 7743

Determining Relative Clinical Potency

Relative clinical potency may be determined in 2 ways. The first is by a literature comparison of the effective doses of each of the products used. This has yielded ratios of Botox/Dysport potency of 4–5:1 (Brin and Blitzer, 1994; Marsden, 1994) or 3–5:1 (Moore, 1995). However, these estimates are not valid because one is comparing different injectors using different techniques on different patients and assessing results with different outcome measures. For example, using an EMG-guided technique, the mean effective dose of Dysport used by for cervical dystonia was 200 MU (Brans *et al.*, 1995). If this figure is compared with the average dose of Botox used, 100–150 MU, a ratio of only 1.5–2:1 is produced. Despite the limitations of the literature comparison approach, when one considers that the minimum effective dose for cervical dystonia of BTX-B in a recent study was 2400 MU (ABCD Study Group 1995), the point is once again made that the LD_{50} assay is an unreliable guide to clinical effectiveness.

The other method is direct comparison whereby the same injectors using the same techniques and clinical measures, and sometimes even the same patients, evaluate the two preparations. There are 2 main approaches. The first is to assume a ratio, for example, 3 or 4:1, and compare the results. The second is to titrate the dose against outcome, that is, use increasing doses until a set outcome is achieved. This could be the minimum effective dose, the maximum effective dose or the dose that produces the same (satisfactory) outcome as had previously been achieved with the other preparation. Direct comparison is more logical than literature comparison but it is still limited by methodological and technical factors. For example, it is not known whether the 2 products have the same stability after being reconstituted so that by the end of a treatment session the relative loss of potencies may be different. Furthermore, each direct comparison approach has its own limitations. The assumption of a fixed ratio does not determine that the ratio chosen is the only

Table 1. Studies directly comparing Dysport and Botox

	Condition	Ratio Dysport/Botox
Whurr *et al.* 1995	Laryngeal dystonia	3:1
Marion *et al.* 1995	Blepharospasm	3:1*
Durif *et al.* 1995	Blepharospasm	6:1
	Hemifacial spasm	(5.3:1*)
Sampaio *et al.* 1996 (in press)	Blepharospasm	
	Hemifacial spasm	4:1

* = same patients received each type of BTX

one which would produce equivalent clinical outcomes. For some conditions there is quite probably a range of effective and tolerated doses, that is, a wide therapeutic window. Likewise, the titration method is complicated by the potential cumulative effects of the toxin. Finally, there are issues of placebo control, randomisation, equivalence of the comparison groups in terms of clinical characteristics, and the validity of outcome measures.

There have been 4 direct comparison studies of Dysport and Botox published (Table 1). Two assumed a ratio (3 or 4:1) and two titrated the dose against outcome. The study of laryngeal dystonia found that a ratio of 3:1 (Dysport dose to Botox dose) produced roughly equivalent clinical outcomes (Whurr *et al.*, 1995). However, the others found ratios ranging from 3 to 6:1 (Durif *et al.*, 1995; Marion *et al.*, 1995; Sampaio *et al.*, in press). The ideal methodology for direct comparison would involve the same injectors using the same techniques. The patients would be clinically equivalent and randomised to the treatment groups which would include doses of each formulation in a range of assumed ratios (3–6:1), and placebo-controls. The whole procedure would be double-blind and use objective, quantifiable and validated outcome measures. Furthermore, it would probably be necessary to perform separate studies for each clinical condition treated, as there is nothing to suggest that the ratio will be the same for laryngeal dystonia as for writer's cramp, for example. Such studies are unlikely to happen.

Possible Explanations for lack of Bioequivalence

The lack of clinical bioequivalence of the two formulations whose potency has been determined by a bioassay has intrigued clinicians because it seems to defy logic. There are 3 possible explanations. Firstly, the LD_{50} mouse assay used for each formulation may not be equivalent. It is well-known that although the assay may be highly reproducible within one laboratory (±5%, Pearce *et al.*, 1994) there is considerable variability between laboratories. Using the UK (Dysport) LD_{50} method, Hambleton and Pickett (1994) found that a vial of Botox (labeled 100 MU) assayed at around 300 MU and a vial of Dysport (labeled 500 MU), around 500 MU. They attributed the differences in clinical potency to differences in the LD_{50} technique used by each company such that the Botox assay underestimates its potency by a factor of about 3. This factor is compatible with the 3–5 × range observed clinically. In a similar comparison, Pearce *et al.* (1994) found similar results but determined a lower ratio of 1.9.

Inter-laboratory LD_{50} variability has been shown also for other serotypes of BTX. A Japanese preparation of BTX-F was assayed at the Chiba Institute as 2405 MU/vial (Ludlow *et al.*, 1992). When re-tested with an LD_{50} assay at NIH it was found to be around 4000 MU/vial (range 3529–4746 MU; Ludlow *et al.*, 1992) whereas at Columbia–Presbyterian Hospital it was assayed at 2520 MU/vial (Green and Fahn, 1993).

The second possibility is in the intrinsic variability of the potencies from one batch to the next. This may be up to ±25% (Sampaio *et al.*, in press). Thus, Dysport might range from 375–625 MU/vial and Botox, 75–125 MU/vial. In a direct comparison, the apparent relative potencies could, theoretically, range from 3–8.3 ×.

The third possibility is that lethal potency (LD_{50}) is not equivalent to paralytic potency. What kills a mouse may involve mechanisms other than paralysis; we know that BTX has autonomic anticholinergic effects, for example. Furthermore, controlled paralysis is the clinical objective, not death! In an elegant study, Pearce *et al.* (1994) showed that amounts of Dysport and Botox that are equivalent in LD_{50} terms do not have equivalent paralytic effects in vivo (mouse hindlimb paralysis). Botox was more potent by 2.4, in addition to the factor of 1.9 × determined by the LD_{50} assay comparison. This brought the total difference in potency to 4.3 × (Botox more potent that Dysport), which is again within the range determined by clinical comparisons.

Relationship Between Weakness and Clinical Response

It is the hope of clinicians that standardisation of the laboratory measures of potency will produce clinically bioequivalent products. However, this assumes that the clinical effect is directly related to one or both of the two current measures of biological potency (lethality or paralysis) and ignores the large number of clinical and technical variables that influence the clinical response, possibly differently for different preparations of BTX.

Table 2. Evidence of additional mechanism of benefit of BTX

Clinical

Dissociation in time and degree

Time
- immediate onset of relief
- clinical benefit outlasting weakness
- clinical benefit beginning well after weakness

Degree
- substantial benefit with minimal weakness
- minimal benefit with marked weakness

Reduced dystonia at sites remote from those injected
Reduced blinking *rates* in blepharospasm
Increased remission rates?

Electrophysiological

Changes in
- evoked potentials (Giladi *et al.*, 1995)
- spinal reflexes – reciprocal inhibition (Berardelli)
- spindle afferent discharge (Manni *et al.*, 1989, Filippi *et al.*, 1993)

Table 3. Clinical factors influencing the biological effects of BTX injected

External (injection technique)
- dose
- dilution
- intramuscular location (motor point)
- accuracy of placement
- spread outside injected muscle
- extramuscular location (blepharospasm)
- number of injection sites

Internal (patient variables)
- individual patient sensitivity – wide variability
- Particular muscle sensitivity
- Abnormal resistance
  - humoral (antibodies)
  - non-humoral
- Amount of dystonic activity? (Hallett *et al.*, 1994)

BTX is used clinically for conditions with excessive muscle contraction because it is possible to produce a focal and graded degree of weakness. While there is no doubt that both weakness and clinical response are dose-dependent, some evidence suggests that there may be another mechanism by which BTX produces its clinical benefit, in addition to neuromuscular junction blockade (Table 2). The importance of this point is that laboratory measures of lethality and paralysis would not reflect this other, unknown mechanism and thus would not necessarily predict clinical response. A non-neuromuscular junction mechanism could also play a role in the different clinical potencies of Dysport and Botox.

Factors Influencing Biological Response to BTX

Clinically it is helpful to distinguish biological response to botulinum toxin from clinical response. Biological response refers to the ability of the toxin to produce NMJ blockade with consequent weakness and atrophy. Clinical response refers to any subsequent improvement in the clinical condition. This distinction is particularly useful when dealing with primary or secondary non-responders (Sheean and Lees, 1995). Even if a laboratory standard by which to measure paralytic potency could be achieved, several clinical and technical factors affect the biological response, that is, the degree of weakness produced in the injected patient.

Factors determining the degree of weakness are of 2 kinds (Table 3), internal (within the patient) and external. Internal factors include individual sensitivity, abnormal resistance, the particular muscle to be injected, amount of muscle activity (normal and abnormal) after injection.

It is the experience of many injectors that individual sensitivity to BTX varies greatly. The dose required to produce a given level of weakness in one subject can vary over a wide range. For example, Pullman (1992) lists from his clinic the range of doses of Botox injected into extensor indicis as 2.5–20 MU and into flexor carpi ulnaris as 5–95 MU. The basis of this variation in individual sensitivity is not known; it could be related to the mechanism by which some patients develop biological resistance to the toxin in the absence of antibodies. Furthermore, the range of individual sensitivity seems to depend upon the muscle injected. In the same table, Pullman (1992) lists the range in biceps as only 25–100 MU.

It is also apparent that the sensitivity of individual muscles *within* the same subject varies widely (Pullman, 1994). This appears to depend largely upon the size of the muscle but functional anatomy and limb biomechanics, as well as relative innervation ratio and end-plate density, may also be important (Pullman, 1994). For example, it is clear that muscles in the extensor compartment of the forearm seem disproportionately sensitive compared with the flexor group. For some muscles, gender is a factor (Pullman, 1994).

It is known that experimentally the amount of muscle activity after injection influences the degree of weakness produced (Eleopra *et al.*, 1993). It has been suggested that if the toxin is drawn to active muscle fibres and if these are active because of dystonia then those fibres involved in the dystonic movements will be preferentially weakened (Hallett *et al.*, 1994).

However, in another study, a better response was obtained after injections for laryngeal dystonia if the voice was rested following the injections (Wong *et al.*, 1995).

Finally, secondary clinical resistance may occur in up to 10% of patients treated with BTX (Greene *et al.*, 1994). In many cases this is associated with biological resistance to BTX due to the presence of neutralising antibodies but not always (Greene *et al.*, 1994; Sheean and Lees, 1995). The level of any such abnormal biological resistance will affect the degree of weakness produced.

The external factors (Figure 1) that influence the degree of weakness, other than dose, relate mainly to technique. Although dilution is listed, animal studies suggest that a 100-fold change in concentration is required to change the degree of paralysis by a factor of 2 (Shaari and Sanders, 1993). Possibly the most important factor involves the injection technique, in particular, the accuracy of placement of the toxin within the muscle. Some groups attribute their ability to use much lower doses of toxin successfully to their use of electromyography (EMG) to accurately guide the injections (Brans *et al.*, 1995). Diffusion of the toxin does not appear to be different between Dysport or Botox (Holt and Fredericks, 1995). Another important factor relates to the degradation of the toxin potency by inappropriate storage or reconstitution (e.g. shaking), the practice of leaving toxin out for many hours after reconstitution and the re-use of toxin previously reconstituted and frozen.

One illustration of the variation in the degree of weakness that is possible comes from test injections of BTX into the extensor digitorum brevis muscle (EDB), usually given to check for biological resistance. In one study, 40 MU of BTX-F (equivalent to 40 MU of Dysport BTX-A) reduced the muscle response amplitude to nerve stimulation to 5–10% of baseline at 4 weeks (Sheean and Lees, 1995). However, in another study *200* MU of BTX-A (Dysport) produced a reduction in the size of the muscle response to 25% of baseline after 4 weeks (Kessler and Benecke, in press). On the other hand, a dose of 10 MU of Botox (roughly "equivalent" to 30–50 MU of Dysport) resulted in a reduction to only 40%, maximum at 3 weeks (Hamajian and Walker, 1994).

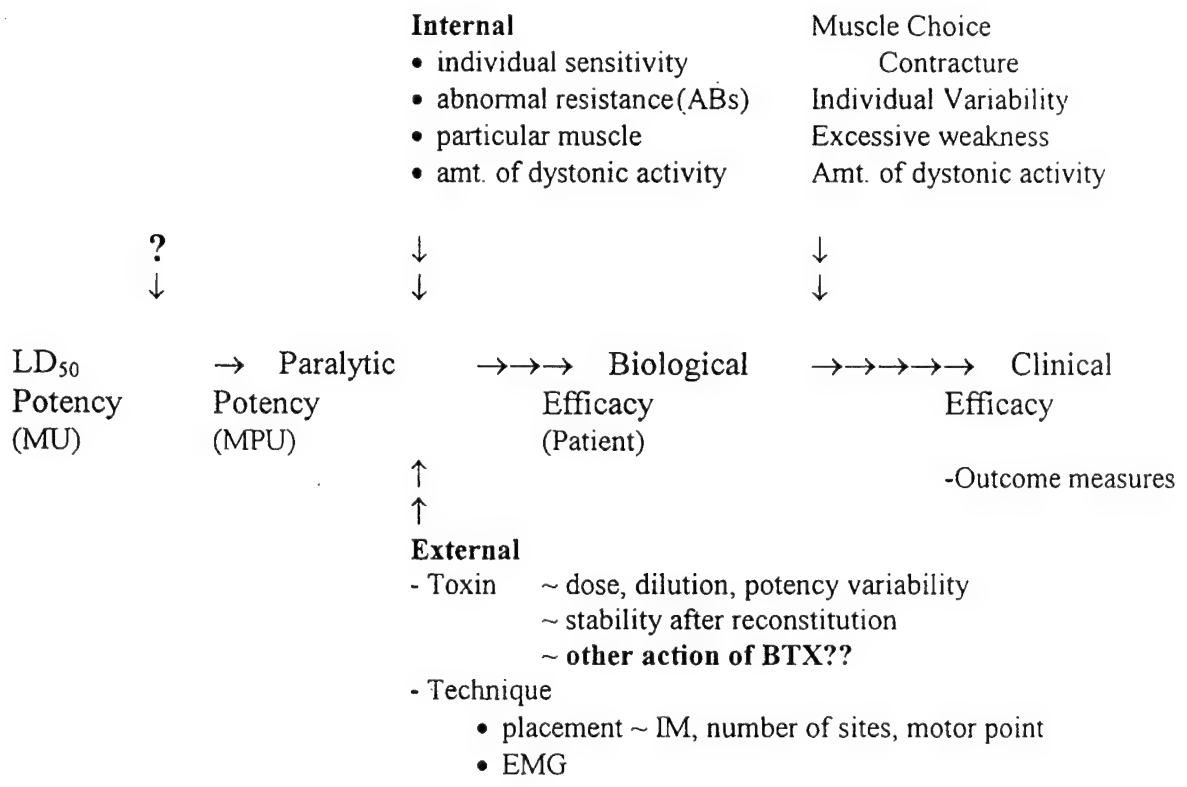


Figure 1. Overview of the sequence from laboratory measures of biology potency to clinical outcome and the factors that influence each step. MU = mouse units; MPU = median paralysis unit (Pearce et al., 1994); IM = intramuscular; ABs = antibodies: EMG = electromygrophy.

Factors Determining Clinical Response to BTX

For a given degree of weakness, there are certain factors that determine the clinical outcome. Possibly the most important is the choice of muscles for injection. Many apparent failures are due to injection of inappropriate muscles. Some muscles may be deep and inaccessible. Even if correct muscles are injected initially, dystonia is dynamic and the pattern of muscles may change after BTX (Gelb *et al.*, 1991) so that the initially injected muscles may no longer be correct. Contractures within the muscle will obviously limit the effectiveness of treatment. The dose-response relationship appears to vary in different muscles. Excessive weakness in limb muscles, particularly the upper limbs (e.g. writer's cramp), can result in a lack of clinical improvement or even a worsening of function that will appear to be a treatment failure. This is less likely in cervical dystonia. The dose of BTX used in the treatment of cervical dystonia is much less now than initially (Greene *et al.*, 1994; Poewe and Wissel, 1994) whereas the success rate is the same. This suggests that for cervical dystonia at least the clinical effect may reach a plateau despite increasing doses. In other words, there is a range of doses which will produce acceptable results representing a wide therapeutic window. Finally, the degree of weakness that is necessary to produce the same *clinical* outcome also appears to vary from patient to patient and from muscle to muscle. Some patients require large amounts of weakness to overcome a dystonic movement whereas others need only a small amount. A point that is particularly relevant to clinical comparison studies is the choice of outcome measure that is used to gauge the clinical response. Finally, there may well be some additional mechanism by which BTX produces a clinical effect that may not parallel the degree of weakness. This as yet undetermined mechanism may also be affected by the same internal and external factors governing the NMJ blockade.

In conclusion, LD_{50} determined potency may not predict paralytic potency, paralytic potency may not predict the degree of weakness produced in the patient, and the degree of weakness may not predict the clinical response. The purpose of elaborating upon all the factors that influence the 2 basic parameters of weakness and clinical response (summarised in Figure 1) was to emphasize this point. If the standard laboratory potency measure adopted does reduce the ratio of relative clinical potencies, then this would be helpful to minimise the degree of potential clinical errors. However, attempts to standardise biological potency in the laboratory will not necessarily result in improved ability to predict clinical response. It is likely that this will still vary from individual to individual, from muscle to muscle and from condition to condition. Furthermore, it is not known how the different formulations and types of BTX will each be affected by the many clinical and technical factors, some of which may still be unknown and uncontrollable. As new types of BTX are brought into clinical practice the problem of lack of bioequivalence is likely to escalate.

References

American BotB cervical dystonia (ABCD) Study Group (1995) BotB (Botulinum toxin type B) in the treatment of cervical dystonia (CD) – protocol AN072-008: an interim analysis (abstract) *Mov Disorders* 10, 372.

Brans JWM, de Boer IP et al. (1995) Botulinum toxin in cervical dystonia; low dosage and EMG-guided application *J Neurol* 242, 529–534.

Brin MF and Blitzer A (1994) Botulinum toxin: dangerous terminology errors (letter) *J Royal Soc Med* 86, 493–494.

Durif F (1995) Clinical bioequivalence of the current commercial preparations of botulinum toxin *Eur J Neurol* 2, 17–18.

Eleopra R, Tugnoli V et al. (1993) The role of activity in the neuromuscular block induced by botulinum toxin (abstract) *Electroencephalogr Clin Neurophysiol* 87, S46.

Filippi GM, Errico P et al. (1993) Botulinum A toxin effects on rat jaw muscle spindles *Acta Otolaryngol* 113, 400–404.

Gelb DJ, Yoshimura DM et al. (1991) Change in the pattern of muscle activity following botulinum toxin injections for torticollis *Ann Neurol* 29, 370–376.

Giladi N, Christyyakov A et al. (1995) The effect of botulinum toxin treatment for craniocervical dystonia on brainstem auditory and trigeminal evoked potentials (abstract) *Mov Disorders* 10, 364.

Greene P, Fahn S et al. (1994) Development of resistance to botulinum toxin type A in patients with torticollis *Mov Disorders* 9, 213–217.

Greene PE and Fahn S (1993) Use of botulinum toxin type F injections to treat torticollis in patients with immunity to botulinum toxin type A *Mov Disorders* 8, 479–483.

Hallett M, Glocker FX et al. (1994) Mechanism of action of botulinum toxin (letter) *Ann Neurol* 36, 449–450.

Hamajian JA and Walker FO (1994) Serial neurophysiological studies of intramuscular botulinum-A toxin in humans *Muscle Nerve* 17, 1385–1392.

Hambleton P and Pickett AM (1994) Potency equivalence of botulinum toxin preparations (letter) *J Royal Soc Med* 87, 719.

Holt D and Fredericks S (1995) Therapeutic botulinum toxin: localisation of proprietary formulations (abstract) *Mov Disorders* 10, 385.

Kessler KR and Benecke R (in press) The "EDB" test – a clinical test for the detection of antibodies to botulinum toxin type A *Mov Disorders*.

Ludlow CL, Hallett M et al. (1992) Therapeutic use of type F botulinum toxin *New Engl J Med* 326, 349–350.

Manni E, Bagolini B et al. (1989) Effect of botulinum toxin on extraocular muscle proprioception *Doc Ophthalmol* 72, 189–198.

Marion MH, Sheehy M et al. (1995) Dose standardisation of botulinum toxin *J Neurol Neurosurg Psychiatry* 59, 102–103.

Marsden CD (1994) Botulinum toxin: dangerous terminology errors (reply) *J Royal Soc Med* 86, 494.

Moore AP (1995) General and clinical aspects of treatment with botulinum toxin. In Moore AP (ed): *Handbook of Botulinum Toxin Treatment*. Oxford: Blackwell Science Ltd.; pp 28–53.

Pearce LB, Borodic GE et al. (1994) Measurement of botulinum toxin activity; evaluation of the lethality assay *Toxicol Appl Pharmacol* 128, 69–77.

Poewe W and Wissel J (1994) Experience with botulinum toxin in cervical dystonia. In Jankovic J and Hallett M (eds): *Therapy*

with Botulinum Toxin. New York: Marcel Dekker Inc.; pp 267–278.

Pullman S (1992) Treatment of limb dystonia with botulinum toxin. *The Clinical Use of Botulinum Toxin:* Course Workbook. New York, November 2nd 1992.

Pullman S (1994) Limb dystonia: Treatment with botulinum toxin. In Jankovic J and Hallett M (eds): *Therapy with Botulinum Toxin*. New York: Marcel Dekker Inc.; pp 307–321.

Quinn N and Hallett M (1989) Dose standardisation of botulinum toxin (letter) *Lancet* i, 964.

Sampaio C, Ferreira JJ et al. (in press) Dysbot: a single-blind, randomised parallel study to compare the efficacy and tolerability of two formulations of botulinum toxin type A: Dysport versus Botox *Mov Disorders*

Schantz EJ and Johnson EA (1990) Dose standardisation of botulinum toxin (letter) *Lancet* 335, 421.

Shaari CM and Sanders I (1993) Quantifying how locations and dose of botulinum toxin injections affect muscle paralysis *Muscle Nerve* 16, 964–969.

Sheean GL and Lees AJ (1995) Botulinum toxin F in the treatment of torticollis clinically resistant to botulinum toxin A *J Neurol Neurosurg Psychiatry* 59, 601–607.

Whurr R, Brookes G et al. (1995) Comparison of dosage effects between the American and British botulinum toxin A products in the treatment of spasmodic dysphonia (abstract). *Mov Disorders* 10, 387.

Wong DL, Adams SG et al. (1995) Effect of neuromuscular activity on the response to botulinum toxin injections in spasmodic dysphonia *J Otolaryngol* 24, 209–216.

The bioactivity of botulinum toxin formulations

L. Bruce Pearce[1,2], Eric R. First[2], Amit Guptab and Robert D. MacCallumb

[1]Associated Synapse Biologics, 11 Hurley Street, Cambridge, Massachusetts
[2]Department of Pharmacology, Boston University School of Medicine, Boston, Massachusetts

Key Words: LD50, MPU, Duration, Denervation, Regional, Potency, Standardization, Clinical, Dysport, Botox

Characterization of the biological activity of clinical formulations of botulinum toxin has been problematic as illustrated by the difference in the reported clinical potencies of two type A toxin formulations, Botox® and Dysport®. This discrepancy is due to problems with the definition and appropriateness of the measures of activity used to define the activities. Our approach to this problem has been to identify an assay that measures the biological action which underlies the therapeutic effect of the toxin and imitates the mode of administration. This involved the use of the mouse hind limb to measure the extent of regional denervation produced following intramuscular administration. Regional denervation was measured by determining the number of mice with hind limb paralysis following intramuscular administration of the toxin. The amount of toxin producing complete hind limb paralysis in 50% of mice was defined as the median paralysis unit, MPU. Following correction for the differences in LD50 units, the potency of these formulations was compared in terms of the regional denervating activity in each formulation. This analysis showed that the denervating activity differed by a factor of 2.4 and that a number of factors contribute to the differences in clinical potency. These and other issues regarding characterization of clinical formulations will be discussed.

The characterization of the bioactivitity of botulinum toxins has evolved from a focus on the toxic (lethal) effects of the toxin to analysis of the more discrete effects such as the proteolytic cleavage of specific proteins within nerve terminals and neurosecretory cells (Van Ermengen, 1897; Schiavo *et al.*, 1992). This has been driven, in part, by the changing medical perspective from that of toxicology to pharmacotherapy and the importance of the neurotoxins as tools for exploring the mechanisms underlying neurosecretion. However, the advances in our understanding of the basic pharmacologic properties of the botulinum toxins has far outpaced the evolution of accepted methods for quantifying the activity of clinical formulations. Since the 19th century the biological activity of botulinum toxins has been detected and measured in terms of lethality. The first experiments involved feeding animals contaminated samples and extracts and then watching for the signs of botulism, including death. Today, botulinum toxin activity is almost universally expressed in terms of the amount of toxin producing 50% lethality in a population of laboratory mice, the mouse LD50. This measure can be applied to all seven serotypes (A, B, C1, D, E, F, and G) of botulinum neurotoxin and for many years has represented the unofficial 'gold standard' for estimating the biological activity.

A major change in our perspective on BoNT occurred as a result of the pioneering work of Dr. Alan Scott in collaboration with Professor Edward Schantz that ultimately led to FDA approval in 1989 of a type A toxin, Botox® (originally Oculinum®), for clinical use in the US. This was followed by approval in the UK of another type A toxin, Dysport® in 1991. It became evident very quickly that there was a marked discrepancy in the clinical potency of these two type A toxin formulations with apparently 3 to 5 times more Dysport® than Botox® (LD50 units) needed to treat the same clinical indications (Quinn and Hallet, 1989; Borodic *et al.*, 1991; Hambleton, 1991; Jankovic and Brin, 1991; Clarke, 1992). The striking and unexplained difference in the clinical potencies of the two formulations prompted our laboratory to compare the biological activity contained in the vials of Dysport® and Botox® formulations. Our analysis of these two formulations

Corresponding Author: L. Bruce Pearce, Ph.D., Associated Synapse Biologics, 11 Hurley Street, Cambridge, Massachusetts 02141. Tel: 617-234-6535; Fax: 617-234-6505.

Table 1. Comparison of Type A Toxin Formulations

Formulation	Units[a]	Correction[b]	MPUs	Ratio
Botox®	100	1.23	297	0.9
Dysport®	500	0.66	331	

[a]Labeled amount of toxin in mouse LD50 units
[b]Correction factor determined by actual units in vials

showed that 66% fewer LD50 units of toxin where found in the Dysport® vials than were indicated on the label (500 U) which was the opposite of what was found for the Botox® formulation which contained 27% more LD50 units than indicated on the label (100 U) as shown in Table 1 (First *et al.*, 1994, Pearce, *et al.* 1994a,b). On the basis of these observations approximately a two-fold adjustment in the relative number of LD50 units in these vials was required to correct for the disparity in the units. More revealing however, were the results observed following intramuscular injection and the measurement of regional denervation/paralysis (Pearce *et al.*, 1995). These experiments showed that 2.4 times more Dysport® was required to produce the equivalent amount of regional denervation observed with Botox®as shown in Table 1. This marked difference in potency was observed even after correction for the number of LD50 units in these vials. Thus, even under equivalent assay conditions the LD50 unit did not predict the amount of regional denervating activity of a given preparation of toxin. Furthermore, these data indicate that neither methodological nor species differences can be used to explain the lack of equivalence of the intramuscular potency of Dysport® and Botox® (Pearce *et al.*, 1994b).

Over the years a number of attempts have been made to establish the LD50 as the fundamental unit of toxin activity. One approach has been to standardise the LD50 measurement against a known standard preparation of toxin (Schantz and Kautter, 1977; Schantz and Johnson, 1990). Other approaches have suggested standardising the way in which the LD50 assay is carried out may resolve the problems with standardization (Pickett and Hambleton, 1994, Hambleton and Pickett, 1994). However, it is clear that even with appropriate standardization of the LD50 unit of activity that this will not resolve the insensitivity of this assay to properties of the toxin or its formulation that contribute to determining clinical potency. It is time to do away with trying to 'fix' the LD50 assay and adopt a new assay that more accurately reflects the chemodenervating effects of the toxin (Pearce *et al.*, 1994b, Sesardic *et al.*, 1994). In order to accomplish this, the new assay must meet appropriate standards for; precision, tolerance, accuracy, sensitivity, and ruggedness. Sesardic *et al.* (1996) have described and evaluated a modification of a regional ptosis assay using the mouse that was originally described by Takahashi *et al.* 1990 which may be of value in evaluating clinical formulations. However, any new assay adopted for this use must be able to predict clinical potency in an equivalent fashion across the board. That is, one unit of activity must always equal the same amount of regional denervating activity irrespective of which toxin is being tested. The first obvious test of any new standard assay for the measurement of toxin activity would be to resolve the differences between Dysport® and Botox®. In this regard, the mouse hind limb paralysis assay has proven an appropriate method for the purposes of characterization of the clinically relevant toxin activity (Pearce *et al.* 1995). A number of factors contribute to the applicability of this method for characterization of the clinically relevant activity of toxin formulations. First, this MPU assay measures the same physiological effect responsible for the therapeutic response to the toxin in the same species used originally to determine the LD50 units. This has helped to reconcile the difference in clinical potencies of Botox® and Dysport®. In addition, there is literature demonstrating the utility of various hind limb preparations of the mouse or rat for studying the actions of the toxin. Accordingly, it is possible to obtain histological and electrophysiological data using this same preparation which allows for the direct correlation with the functional effects of the toxin.

There are a number of measures of botulinum toxins that can be used to estimate potencies, however the factors contributing to the clinical pharmacologic potency of botulinum toxin paint a complex and to a certain extent a not very well characterized picture. To appreciate the complex nature of the pharmacologic activity of botulinum toxins we need to briefly survey the steps and processes that are thought to contribute to the extent and duration of the effect of the toxin on neurotransmitter release following intramuscular injection (Figure 1). As noted above the potency of various preparations of BoNT following intramuscular injection are different and while the mechanism(s) responsible for these differences have not been established, it is likely that this is related to differences in the stability of the toxins once injected into muscle. (Pearce *et al.*, unpublished results; Sesardic *et al.* 1996). Thus, one factor that determines potency, the stability of BoNT in muscle tissue, can be expressed in terms of an extracellular half-life, t1/2extra, the shorter the half-life the lower the potency.

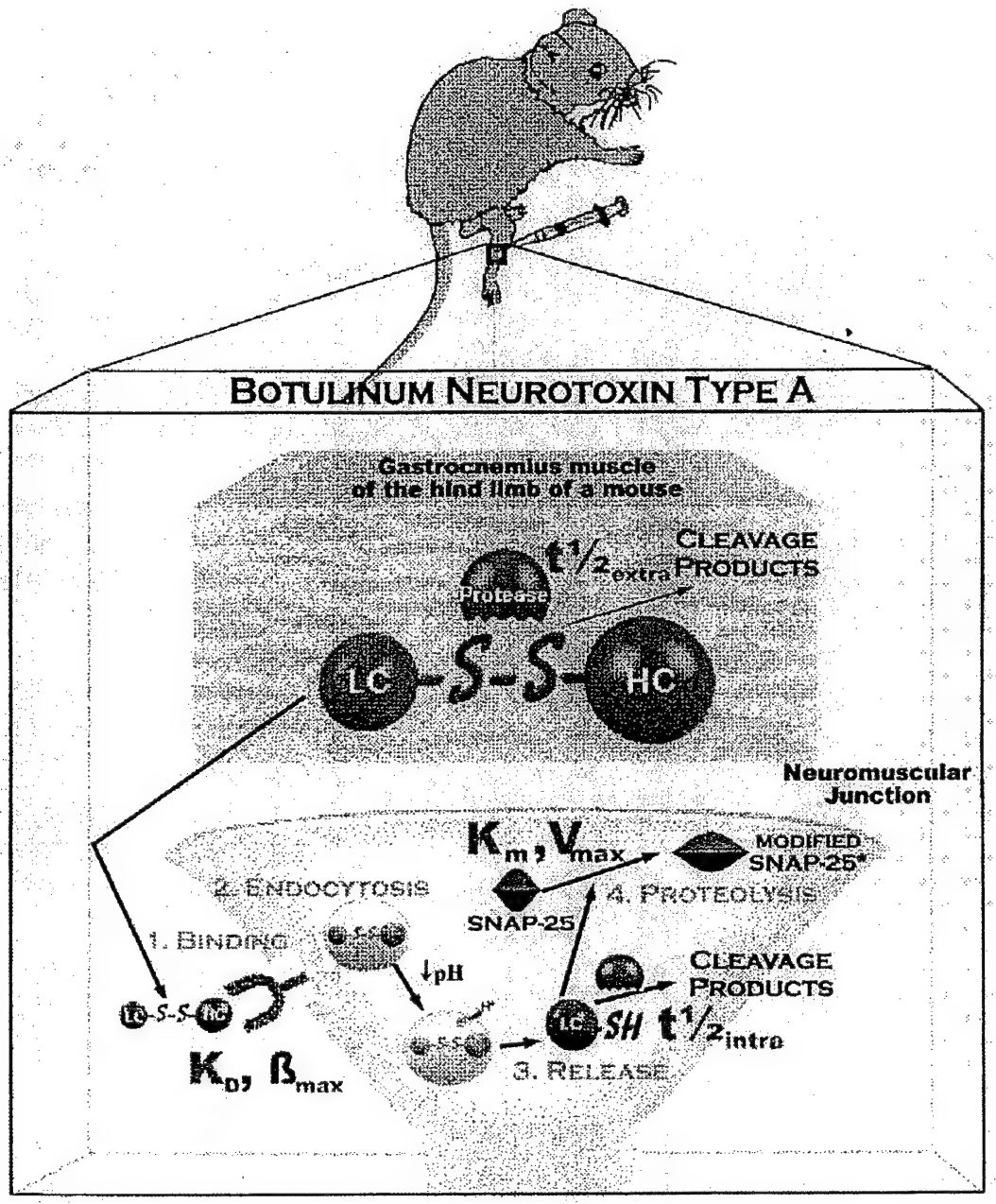


Figure 1. Toxin serotype specific factors affecting the potency of botulinum toxin formulations. This figure illustrates some of the factors affecting the potency of botulinum toxin administered to muscle. Following intramuscular injection, the toxin may be destroyed by endogenous proteases before it can reach the neuromuscular junction. Thus the toxin has a certain extracellular half-life, $t_{1/2extra}$. Once the toxin diffuses to the neuromuscular junction it binds to its acceptor/receptor and enters the nerve terminal via what has been referred to as an endocytotic mechanism. This unique binding step can be described for each of the toxin serotypes by the Kd and βmax constants. Upon acidification of the endosome the disulfide bond linking the heavy and light chains is thought to be reduced. The heavy chain is hypothesized to form a pore in the endosome membrane and provide a means by which the reduced light chain can gain access to the intraterminal cytosol. The light chain of the toxin may have a finite lifetime within the nerve terminal, $t_{1/2intra}$, determined perhaps by neuronal protease inactivation. Once free within the nerve terminal cytosol the toxin light chain selectively cleaves specific peptide bonds of proteins (SNAP-25, syntaxin, synaptobrevin/VAMP) critical to the neurotransmitter release process. The endopeptidase activity of the light chain can be defined by the substrate Km and Vmax.

Once the neurotoxin reaches the neuromuscular junction there are still a number of steps required before the toxin can express its biological activity (Simpson, 1981). The toxin binds to a receptor/acceptor at the nerve terminal and the pharmacologic potency of this binding step in the process can be described by the dissociation constant, Kd, for the large molecular weight subunit for its receptor/acceptor. Another receptor related parameter that contributes to the potency is the receptor number, bmax, this determines the number of toxin molecules that enter the nerve terminal. Subsequent to the toxin binding to the receptors on cholinergic nerve terminals at the neuromuscular junction the toxin enters the nerve terminal by endocytosis (Simpson, 1981). Upon acidification of the resulting endosome it is thought that the disulfide bond coupling the heavy and light chains of the toxin is reduced and via a pore formed by the heavy chain in the endosome membrane the light chain then gains access to the intraterminal cytosol. In addition to extracellular tissue stability, the results of studies conducted in adrenal chromaffin cells (Bartels *et al.*, 1994) suggest that the intracellular half-life, $t_{½intra}$, of the toxin is another important factor in determining the duration of action.

Once free to move within the nerve terminal the light chain of the toxin cleaves a particular substrate protein including synaptobrevin/VAMP, SNAP25, or syntaxin 1 (Schiavo *et al.*, 1992; Blasi *et al.*, 1993a,b). The particular protein cleaved by the endopeptidase activity of the light chain depends on the particular toxin serotype. The light chains for the specific serotypes (A–G) probably possess unique affinities for their substrate proteins. Accordingly, the Km and Vmax for these specific substrates are constants that can be used to characterize this component of the pharmacologic activity of the light chains. Thus, there are at least six constants that are probably unique for each toxin serotype that are needed to characterize the intramuscular pharmacologic activity of BoNTs: $t_{1/2extra}$, $t_{1/2intra}$, Kd, βmax, Km, and Vmax. It should be clear from this discussion that a complex combination of factors contributes to the intramuscular potency of the toxins and careful characterization of one of these parameters is probably not sufficient to characterize the pharmacologic potency in all situations.

Each of these parameters that can be defined for the toxin may be perfectly valid (perhaps the only valid measures) for characterization of specific properties of the toxin, however, the appropriateness of each of the measures is limited by the point of reference of the investigator. Some measures of toxin activity, like the LD50, apparently incorporate some but not all of these factors, but is perfectly valid for the toxicologist interested in the lethal properties of the toxin. The clinician derives little useful information about what dose of toxin to administer to a patient from the physical constants like the Kd and Km and is likely to be much more interested in a measure of the regional denervating activity (MPU), which incorporates all of these constants contributing to potency.

A critical dimension of the clinical bioactivity of BoNTs that has not been formally characterized is the duration of action. This is perhaps not very surprising considering the unit of activity used to define clinical formulations has been the mouse LD50. This measure of bioactivity has no time dimension and thus provides no information about the persistence of the localized effects of the toxin. Nonetheless, the duration of action of BoNTs is a critical feature of the pharmacologic activity and perhaps one of the most important factors contributing to the clinical utility of this neurotoxin. The toxin would be of little clinical significance if the duration of action was on the order of days or even a week. Thus, when we consider characterization of the clinically relevant pharmacologic activity of BoNTs, we need to consider measures of *both* potency and duration of action. Typically, therapeutic botulinum toxin treatment results in beneficial effects lasting several months. Different formulations of toxin containing different serotypes (A, B or F) have different duration of action when examined clinically. It is becoming widely acknowledged that the type B and F serotypes are not as potent as the type A toxins used clinically with respect to the duration of action (Ludlow *et al.*, 1992; Green and Fahn, 1993; Mezaki *et al.*, 1995, Tsui *et al.*, 1995).

Clearly, inhibition of neurotransmitter release by BoNTs underlies the therapeutic effect. What remains to be defined is exactly which factors or processes are responsible for the reversal of inhibition of neurotransmission and thus, determine the duration of action. One group of observations suggests that the loss of therapeutic effect with time is associated with the re-establishment of muscle innervation as a result of resprouting of neurons (Duchen 1970; Alderson *et al.*, 1991, Borodic *et al.*, 1994). Another possibility is that the half-life of the light chain within the nerve terminal (t1/2intra) is an important factor in determining the duration of action. That is, the light chain of the toxin inhibits neurotransmitter release by cleaving critical proteins involved in neurotransmitter release, but eventually, endogenous proteases within the nerve terminal could cleave the toxin light chain and subsequently the normal release of neurotransmitter would be re-established following the re-synthesis of these proteins needed for normal release. This has been

suggested by recent findings using chromaffin cells in culture as a model system for studying the effects of BoNTs on vesicular release processes. Bartels *et al.* (1994) reported that introduction of specific antibodies to the zinc-binding domain of the light chain of BoNT reversed the inhibition of norepinephrine release from BoNT treated chromaffin cells in culture.

If regeneration of neuromuscular function was due to resprouting alone, then regeneration of the muscle, and return of symptoms, should occur at the same rate for all serotypes. However, as noted above, differences in the duration of therapeutic effect of different serotypes have been observed clinically. One caveat here is that the amount of toxin administered to these patients was based upon the LD50 unit of activity and this does not guarantee that they received the same amount of denervating activity. A more reliable comparison would involve injecting doses of toxin based on units of denervating activity (i.e. MPUs) and then making the comparison of the duration of effect. This has not been done with all of the serotypes, however, experiments performed in mice with type A and B toxins indicate that when the same amount of toxin, measured in terms of median paralysis units (MPUs), is injected into the mouse hind limb that the duration of paralysis is identical for the type A and B toxins as shown in Figure 2. In contrast, when the same number of LD50 units of activity are used the duration of action is not equivalent.

The importance of the duration of action of the

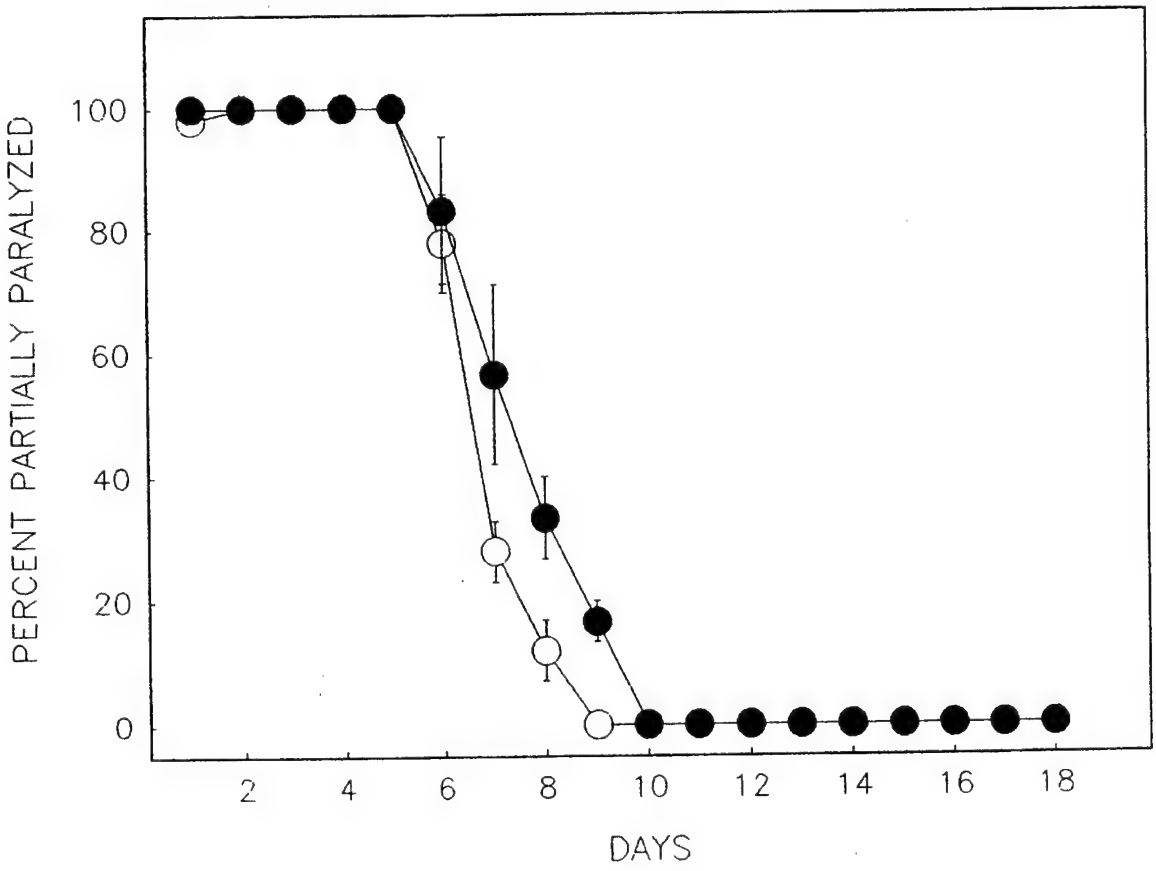


Figure 2. Duration of action of type A and type B botulinum toxin formulations. White CD1 18–22 g male mice (Charles River Laboratories) were administered 0.1 ml (1.0 MPU) of freeze dried formulations dissolved in 0.2% gelatin (type A porcine, Sigma Chemical Co.) in 30mM sodium phosphate buffer, pH 6.2, of either the type A (●–●) or type B (○–○) toxin by i.m. injection into the gastrocnemius muscle of the hind limb. The mice were then monitored daily to determine the presence of absence of complete paw paralysis. The percentage of mice showing complete paw paralysis is presented as a function of time. Five (B toxin) to ten (A toxin) mice were used for each experiment and the error bars represent the S.E.M. from at least three experiments. The maximum duration of action was 9 days for the type A toxin and 8 days for the type B toxin.

regional denervating effect on the clinical pharmacology of the toxin dictates that an additional measure is needed to more fully define the clinically relevant bioactivity. This parameter should clearly define the relationship between duration of action and the units administered in the animal model used to define the units of activity. Using the MPU as an example it is possible to express the ratio of the number of days of regional denervation produced per dose expressed in MPUs. This ratio is 9 or 8 for the type A and B toxins tested as shown by the data in Figure 2. Thus, for a particular denervating dose, which is defined equivalently for all toxins, a measure of the duration of action, a duration of action index, would be provided. If, as mentioned above, the ratio between the duration of action and the dose in regional denervating units is the same for all toxins then there is no need to define such an index. However, it is not clear at this point if this ideal situation holds true or if the relationship between the degree of regional denervation and the duration of action is unique for each of the different serotypes and formulations of the toxin.

Scientists and clinicians interested in therapeutic botulinum toxins are faced with the fundamental problem of identifying the most appropriate definition of the biological activity for a class of proteins that can vary in terms of specific activity and formulation from manufacturer to manufacturer. Thus, physical measures (amount of toxin protein) are very unlikely to provide equivalent information about the biological activity of BoNTs in general. In addition, the biological activity is a function of the route of administration as evidenced by the difference in the potencies observed on im versus ip administration. Therefore, a complex combination of both tissue and toxin-dependent factors contribute to determining the extent and duration of the therapeutic effect. No matter which assay method is finally utilized to characterize the biological activity of therapeutic formulations of the toxin, ideally it should provide a measure of activity that incorporates all of these factors and is valid irrespective of toxin serotype, purity or formulation. It is very important that we resolve this problem because until such time as the biological activity contained in different therapeutic formulations of botulinum toxins is defined in a clinically meaningful way it will be impossible to ensure the content of biological activity in therapeutic formulations. Furthermore, it will remain very difficult to develop a clear understanding of the underlying basis for the fundamental differences in the clinical pharmacology of available formulations and the development of this important and highly efficacious mode of drug therapy will remain retarded.

References

Alderson K, Holds, JB, and Anderson, RL (1991) Botulinum induced alteration in human orbicularis oculi following treatment for blepharospasm *Neurology* 41, 1800–1805.

Bartels F, Bergal H, Bigalke H, Frevert J, Halpern J and Middlebrook J (1994) Specific antibodies against the Zn2+-binding domain of clostridial neurotoxins restore exocytosis in chromaffin cells treated with tetanus or botulinum A neurotoxin *J Biol Chem* 269, 8122–8127.

Blasi J, Chapman ER, Binz T, Yamasaki S, Camilli PD, Sudhof TC, Niemann H and Jahn R (1993a) Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-1/syntaxin *EMBO J* 12, 4821–4828.

Blasi J, Chapman ER, Link E, Binz T, Yamasaki S, De Camilli P, Sudhof TC, Niemann H and Jahn R, (1993b) Botulinum Neurotoxin A Selectively Cleaves the Synaptic Protein SNAP-25 *Nature* 365, 160–163.

Borodic GE, Pearce LB Johnson EA and Schantz EJ (1991) Clinical and Scientific Aspects of Botulinum A *Toxin Ophthalmology Clinics of North America* 4, 491–504.

Borodic GE, Ferrante RJ, Pearce LB and Alderson K (1994) Pharmacology and histology of therapeutic application for botulinum toxin In: Jankovic, J and Hallet, M (eds): *Therapy with Botulinum Toxin* Marcel Dekker Inc. New York.

Clarke CE (1992) Therapeutic Potential of Botulinum Toxin in Neurological Disorders *Quart J Med* 299, 197–205.

Duchen, LW (1970) Changes in motor innervation and cholinesterase localization induced by botulinum toxin in skeletal muscle of the mouse: differences between fast and slow muscles *J Neurol Neurosurg Psychait* 33, 40–54.

First ER, Pearce LB and Borodic GE (1994) Dose standardisation of botulinum toxin *Lancet* 343, 1035.

Green PE and Fahn S (1993) Use of botulinum toxin type F injections to treat torticollis in patients with immunity to botulinum toxin type A *Mov Disord* 8, 479–483.

Hambleton P (1991) Clostridium botulinum toxins: a general review of involvement in disease, structure, mode of action and preparation for clinical use *J Neurol* 239, 16–20.

Hambleton P and Pickett AM (1994) Potency equivalence of botulinum toxin preparations *J R Soc Med* 87, 719.

Jankovic, J and Brin, M (1991) Therapeutic uses of botuliinum toxin *N Engl J Med* 324 1186–1194.

Ludlow CL, Hallet M, Rhew K, Cole R, Shimizu T, Sakaguchi G, Bagley JA, Schulz GM, Yin SG and Koda J (1992) Therapeutic use of type F botulinum toxin *N Engl J Med* 326:349–350.

Mezaki T, Kaji R, Kohara N, Fujii H, Katayama M, Shimizu T, Kimura J and Brin MF (1995) Comparison of the therapeutic efficacies of the type A and F botulinum toxins for blepharospasm. A double-blind controlled study *Neurology* 45, 506–508.

Pearce LB, Borodic GE, Johnson EA, First ER and Mac Callum RD (1994a) Measurement of botulinum toxin: Assessment of the lethality assay *Toxicol Appl Pharmacol* 128: 69–77.

Pearce LB, Borodic GE and First ER (1994b) Botulinum toxin potency: a mystery resolved by the median paralysis unit *J R Soc Med* 87, 571–572.

Pearce LB, Borodic GE, Johnson EA, First ER and MacCallum RD (1995) The median paralysis unit: a more pharmacologically relevant unit of biological activity for botulinum toxin *Toxicon* 33, 217–227.

Pickett AM and Hambleton P (1994) Dose standardisation of botulinum toxin *Lancet* 344, 474–475.

Quinn N and Hallett M (1989) Dose standardisation of botulinum toxin. *Lancet* 1, 964.

Schantz EJ and Johnson EA (1990) Dose standardisation of botulinum toxin. *Lancet* 335, 421.

Schantz EJ and Kautter DA (1977) Microbiological methods: standardized assay for Clostridium botulinum toxins. *J AOAC* 61, 96–99.

Schiavo G, Benfenati F, Poulain B, Rossetto O, Polverino de Laureto P, DasGupta BR and Montecucco C (1992) Tetanus and botulinum B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin *Nature* 359, 832–835.

Sesardic D, Das RE and Corbel MJ (1994) Botulinum toxin *J R Soc Med* 87, 307.

Sesardic D, McLellan K, Ekong TAN and Das RG (1996) Refinement and validation of an alternative bioassay for potency testing of therapeutic botulinum type A toxin. *Pharmacol Toxicol* 78, 283–288.

Simpson LL (1981) The origin, structural and pharmacological activity of botulinum toxin *Pharmacol Rev* 33, 155–188.

Takahashi M, Kameyama S and Sakaguchi G (1990) Assay in mice for low levels of Clostridium botulinum toxin *Int J Food Microbiol* 11, 271–278.

Tsui (1995) Botulinum toxin type B in the treatment of cervical dystonia; a pilot study *Neurology* 45, 2109–2110.

Van Ermengen E (1897) Ueber einem neuen anaeroben Bacillus und seine Beziehungen zum Botulismus. *Z Hyg infektionskrankh* **26**, 1–56 (English Translation: (1979) *Rev Infect Dis* **1**, 701–19.).

The development of novel assays for the detection and quantitation of the botulinum toxins

M. Wictome, K. Newton, B. Hallis and C. C. Shone*

Centre for Applied Microbiology and Research, Porton Down, Salisbury. SP4 OJG, UK.

Key words: *Clostridium botulinum*; ELISA; neurotoxin; peptide substrate; diagnostic test.

A novel assay format for the detection of the botulinum neurotoxins has been developed. The assay for the detection of the type B neurotoxin involves the cleavage of a synthetic peptide substrate representing amino acid residues (60–94) of the intracellular target for the toxin, VAMP (vesicle-associated membrane protein or synaptobrevin) incorporating a C terminal biotin moiety. Neurotoxin is immobilised on a solid-phase consisting of a polystyrene bead coated with a monoclonal antibody (mAb) specific for the B serotype toxin. The VAMP peptide is then added and cleaved by BoNT/B. The resulting peptide fragments are then transferred to a second solid-phase consisting of a streptavidin coated polystyrene microtiter plate. The prescence of the N-terminal cleaved fragment, a result of cleavage of Gln76–Phe77 bond by the toxin is detected by the addition of an antibody that specifically recognises the N terminus of the cleavage product. The assay is able to detect approximately 10 pg/ml of neurotoxin, a sensitivity comparable to the current mouse lethality test, in samples such as serum that prove difficult using standard immunological methods.

The A, B and E serotype neurotoxins produced by *Clostridium botulinum* are the cause of the majority of the reported cases of food-borne human botulism. Botulism, presented as flaccid paralysis, as a result of ingestion of pre-formed toxin present in contaminated foods is often fatal. Currently the only accepted method for the detection of toxin in samples is the mouse bioassay (lethality test) (Gibson *et al.*, 1987). Although highly sensitive (10–20 pg/ml) this test has a number of drawbacks: it is expensive to perform; lacks specificity and involves the use of a large number of animals. With increasing resistance to such animal tests, there is a need to replace the mouse bioassay with a reliable *in vitro* test with a comparable sensitivity. Though a range of immunoassay systems have been reported none are able to match the sensitivity of the mouse bioassay without the need of complicated and expensive amplification systems (Shone *et al.*, 1985; Doellgast *et al.*, 1994)

It has been demonstrated that all the botulinum neurotoxins are highly specific zinc endoproteases which act intracellularly by cleaving proteins involved in the control of neurotransmitter secretion. Botulinum toxins type A (BoNT/A) and E cleave SNAP-25 (synaptosomal associated protein) at two different sites (Schiavo *et al.*, 1993). Botulinum type B cleaves VAMP at a specific site (Schiavo *et al.*, 1992, 1993). A synthetic peptide substrate, representing residues 60–94 of human VAMP isoform-1, for botulinum type B neurotoxin has been defined, which is cleaved with the same specificity as for the *in vivo* target (Shone & Roberts, 1992). Using such synthetic peptide substrates, first generation assay systems, based on the endopeptidase activities of the neurotoxin, have been developed for BoNT/A and BoNT/ B (Hallis *et al.*, 1996). While having the advantage of being rapid and measuring a biological activity of the toxins, such assays, however, have the same sensitivity as current unamplified immunological methods and are not suitable for the detection of toxin present in food and serological samples which potentially contain compounds that interfere with the endopeptidase activity of the neurotoxins. Here we report an assay format, based on the endopeptidase activity of BoNT/B that is able to detect neurotoxin at levels comparable to the mouse test and is applicable to a wider range of sample types.

* Corresponding author. Tel: (44) 1980 612793; Fax: (44) 1980 610848

Materials and Methods

Purification of Botulinum Type B Neurotoxin

Type B neurotoxin (Okra) was purified as described in Shone *et al.* (1993). Biological activity of the toxin was measured using a mouse bioassay as previously described (Gibson *et al.*, 1987).

Synthesis of a Biotinylated Peptide Substrate for BoNT/B (B-VAMP$^{60-94}$)

A peptide corresponding to residues Leu60–Lys94 of human VAMP isoform 1, incorporating an additional C terminal cysteine residue was synthesised as described in Shone *et al.* (1993). The crude product was reacted, overnight at room temperature with a two fold molar excess of N-biotinyl-N′-[6-maleimidohexanoyl]-hydrazide in 50 mM sodium phosphate, 1 mM EDTA, pH 6.0. The peptide was then purified by reverse-phase HPLC, characterised by reaction with dithionitrobenzoic acid and mass spectrometry as described (Wictome *et al.*, 1996). The rate of cleavage of the biotinylated substrate B-VAMP$^{60-94}$ was measured as described (Shone *et al.*, 1993).

Production of Antiserum

Antiserum was produced in guinea-pigs against the peptide FESSAAKC, representing the C-terminal side of the cleavage site on VAMP-1 as described in Hallis *et al.* (1996).

Endopeptidase Assay for Botulinum Type B Neurotoxin

BoNT/B was diluted in 0.5 ml of 50 mM HEPES, 20 μM $ZnCl_2$, pH 7.4 (HEPES/Zn) buffer containing 5% Foetal Calf Serum FCS added to the 1st solid-phase comprised of a polystyrene tube containing a 0.25 inch polystyrene bead coated with two different mAbs specific for the B serotype toxin. After shaking at room temperature for 1 h the bead was washed three times with HEPES/Zn buffer and 100 μl of 100 μM B-VAMP$^{60-94}$ added in HEPES/Zn buffer containing 10 mM dithiothreitol. After shaking at 37°C for 2.5 h the sample was diluted to 500 μl with phosphate buffered saline containing 0.1% Tween-20 (PBS-Tw). Samples (100 μl) were then transferred to the second solid phase consisting of a streptavidin coated microtiter plate. After 5 min, at 37°C, the plate was washed (×3, PBS-Tw) and the cleaved ends generated by neurotoxin were detected by incubation with the antibody specific for the cleaved end followed by an anti guinea-pig immunoglobulin horseradish peroxidase conjugate as described in Hallis *et al.* (1996)

Table 1. (a) Cleavage of peptide (100 μM) by BoNT/B (10 nM) and (b) Effect of human serum on the BoNT/B assay.

(a) *Peptide*	*Rate of Cleavage* (μmoles/min/mg)
VAMP$^{60-94}$	7.8 ± 0.2
B-VAMP$^{60-94}$	10.4 ± 0.1

(b) *Toxin Diluent*	*Concentration to Give 0.5 OD*
Control (Hepes buffer)	~10 MLD_{50}
Human Serum	~10 MLD_{50}

Reaction was monitored by incubation of peptide with toxin, followed by analysis of the fragments by reverse-phase HPLC.
Toxin was diluted as indicated and the concentration to give 0.5 OD above background calculated.

Results and Discussion

A previous study using a range of VAMP peptides has demonstrated that a peptide representing human VAMP isoform 1 (60–94) is cleaved at a similar rate to that observed for larger peptides (Shone *et al.*, 1993). Table 1 shows that the rate of cleavage of the biotinylated substrate, B-VAMP$^{60-94}$ occurs at rate comparable to the unmodified peptide. An assay system involving the cleavage and detection of solid-phase immobilised peptide substrates by neurotoxins A and B has been developed (Hallis *et al.*, 1996) This first-generation assay has the draw-back of a low sensitivity, with a detection limit of around 1–10 ng/ml of toxin corresponding to around 100–1000 MLD_{50} units. It has been estimated that per microtitre well only 10^{-10} mole of peptide can be immobilised; this in conjunction with the high K_m of the toxin for the peptide (0.3 mM) and the inaccessibility of a proportion of bound peptide to toxin results in cleavage of only a small proportion of the peptide (Hallis *et al.*, 1996). This is confirmed using a fluorometric assay which estimates that less than 10% of the total peptide is cleaved after 1 h (data not shown). The exacting assay conditions in terms of ionic strength, pH and presence of reducing agents required to support the endopeptidase activity of the toxins also make the first generation assays impractical for use in a range of food environments. These assays also have difficulty detecting toxin present in serological samples, where high levels of immunoglobulin cause interference (data not shown).

Use of high binding capacity polystyrene beads allows greater absorption of mAb and subsequent toxin to the first solid-phase. The use of mAbs that are specific for the non-enzymatic heavy chain of the toxin ensure that there is no interference with the enzymatic action of the toxin, whilst on the

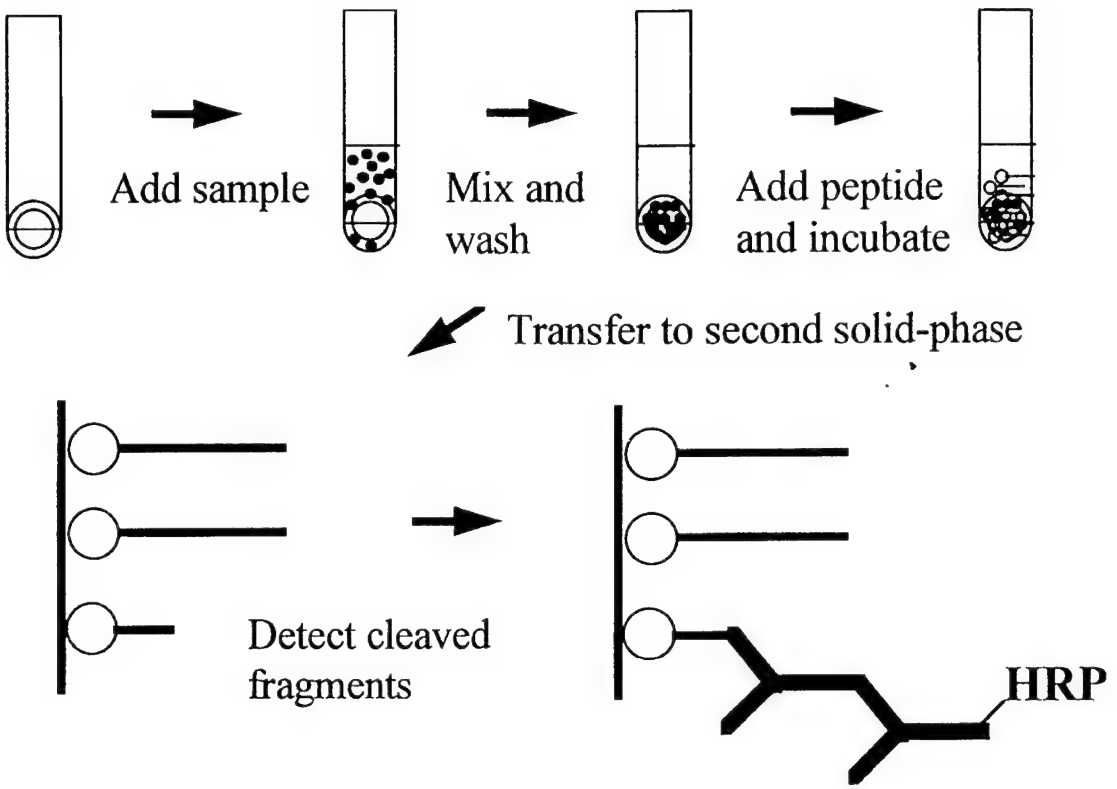


Figure 1. BoNT/B Assay. (See description in Materials and Methods).

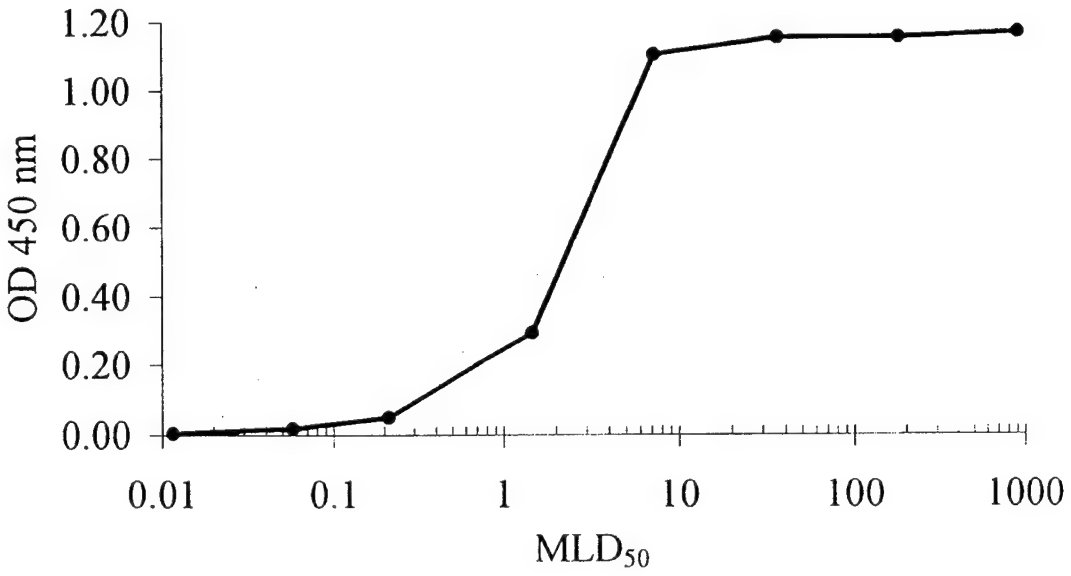


Figure 2. BoNT/B Assay. Assay performed as described in the text. 1 MLD50 was taken as 10 pg/ml. Detection limit. taken as 0.2 OD, above background was 1.03 ± 0.6 MLD_{50} (mean ± standard deviation, n = 15).

solid support. After toxin immobilisation, material in the sample that may affect the endopeptidase activity of the bound toxin can be removed by washing. Such a format allows the assay to be applied to problematic samples such as food and sera. Subsequent addition of biotinylated peptides in concentrations approaching their K_m allows efficient cleavage of the peptide substrate which is then subsequently captured on the second solid-phase and detected using specific antisera. The specificity of both the capture mAbs and that of the cleavage reaction results in an assay that is potentially less prone to false positive results than current immunological methods. The assay, having an equivalent detection limit, may also prove a realistic alternative to the mouse bioassay, Table 1(b), Figures 1,2.

Acknowledgments

This work was supported by a grant (FS1220) from the Ministry of Agriculture, Fisheries and Food.

References

Doellgast GJ, Beard GA, Bottoms JD, Cheng T, Roh BH, Roman, MG, Hall PA & Triscott MX (1994) Enzyme-linked immunosorbent assay and enzyme-linked coagulation assay for detection of *Clostridium botulinum* neurotoxins A, B and E and solution-phase complexes with dual-label antibodies. *J. Clin. Microbiol.* **32**, 105–11

Gibson AM, Modi NK, Roberts TA, Shone CC, Hambleton P & Melling J (1987) Evaluation of a monoclonal antibody-based immunoassay for detecting *Clostridium botulinum* type A toxin produced in pure culture and an inoculated cured meat culture. *J. Appl. Bacteriol.* **63**, 217–226

Hallis B, James BAF & Shone CC (1996) Development of novel assays for botulinum type A and B neurotoxins based on their endopeptidase activities.. *J. Clin. Microbiol.* **34**, 1934-1938.

Schiavo G, Benefati F, Poulain B, Rossetto O, Polverino de Laureto P, DasGupta BR & Montecucco C. (1992) Tetanus and botulinum-B neurotoxins block transmitter release by proteolytic cleavage of synaptobrevin. *Nature (London)* **359**, 832–835

Schiavo G, Rossetto O, Catsicas S, Polverino de Laureto P, DasGupta BR, Benefati F & Montecucco, C (1993) Identification of nerve terminal targets of botulinum types A, D and E. *J. Biol. Chem.* **268**, 23784–23787

Shone CC & Roberts AK (1992) Peptide substrate specificity and properties of the endopeptidase activity of botulinum type B neurotoxin. *Eur. J. Biochem.* **217**, 965–270

Shone CC, Wilton-Smith P, Appleton N, Hambleton P, Modi NK, Gatley S & Melling J (1985) Monoclonal antibody-based immunoassay for type A *Clostridium botulinum* neurotoxin is comparable to the mouse bioassay. *Appl. Environ. Microbiol.* **50**, 63–67

Shone CC, Quinn CP, Wait R, Hallis B, Fooks SG & Hambleton P (1993) Proteolytic cleavage of synthetic fragments of VAMP isoform-2 by botulinum type B neurotoxin. *Eur. J. Biochem.* **217**, 965–971

Wictome M, Rossetto O, Montecucco C & Shone CC (1996) Substrate residues N-terminal to the cleavage site of botulinum type B neurotoxin play a role in determining the specificity of its endopeptidase activity. *FEBS Lett.* **386**, 133–136

Alternative *in vitro* bioassay methods for BoNT/A activity: implications for potency testing of clinical formulations

T. A. N. Ekong, I.M. Feavers, K. McLellan and D. Sesardic

Division of Bacteriology, National Institute for Biological Standards and Control
Blanche Lane, South Mimms, Potters Bar, Herts, EN6 3QG, UK.

Key words: Alternative methods, BoNT/A, zinc endopeptidase, potency testing, clinical formulations

Botulinum neurotoxin type A (BoNT/A) is a powerful toxin which is now being used to treat a number of neuromuscular conditions. There is a need for replacement assays for toxin activity which do not rely on *in vivo* methods, indeed, the currently used bioassay is a priority target for replacement. Most *in vitro* alternative assays are dependent on immunodetection of toxin protein. We have developed a sensitive ELISA assay and shown it to be useful in detecting BoNT/A in clinical formulations. However, there was no relationship between the amount of toxin detected and its biological activity as the ratio of active to total toxin varied for different preparations. This inability to detect active BoNT/A has limited the applicability of ELISAs for potency estimations. A valid alternative assay should be mechanism based. The recent discovery that BoNT/A selectively cleaves SNAP-25, a synaptosomal associated membrane protein, has provided a basis for the development of functional *in vitro* bioassays. Using recombinant methods we have prepared a fragment of SNAP-25 (residues 134–206) spanning the BoNT/A cleavage site as a fusion protein, and have used it as an *in vitro* substrate for the toxin. An immunoassay was developed, using targeted antibodies specific for the C-terminus of intact or cleaved SNAP-25. The assay was used to examine BoNT/A activity in clinical preparations. It is sensitive, with a limit of detection equivalent to 0.2–0.8 mouse LD_{50}/ml (*ie* $<5\%$ of a therapeutic dose), and a gcv of 5.9–11.2% ($n = 15$). It is simple and rapid, and shows a good correlation with the *in vivo* bioassay ($r = 0.93$, $n = 31$). Preliminary results look promising, but the assay will require further validation before it can be recommended for use in potency estimations of clinical preparations of BoNT/A.

The therapeutic use of botulinum neurotoxin type A (BoNT/A) was pioneered by Scott *et al.* (1973) who was the first to use it as a specific localized muscle relaxant for the treatment of focal dystonias. This use has since expanded and intramuscular injection of BoNT/A is now the treatment of choice for a wide variety of neuromuscular conditions (Jankovic and Brin, 1991). Therapeutic formulations contain minute amounts of toxin and non-toxin protein complexed to haemagglutinin with serum albumin added as a bulking agent for stability. For batch release, therapeutic formulations need to be tested to ensure safety, and to establish efficacy and consistency. Currently, the most sensitive method for estimating the activity of this toxin, and the only one which is accepted by the licensing authorities for this purpose is the mouse bioassay with lethality as the end point. However, this technique is cumbersome, time-consuming and requires large numbers of laboratory animals. There is therefore a need for methods which not only reduce the numbers of animals and the severity of the procedures used, but which would ultimately replace the use of animals in these experiments.

In order to be acceptable as a suitable replacement for potency testing of clinical formulations of BoNT/A, an assay would need to be very sensitive (able to detect the minute quantities of toxin in clinical formulations), specific and technically simple so that it may be readily adaptable for routine use. Finally, the assay should be functional and be able to measure biologically active toxin in therapeutic preparations.

Corresponding author: Dr D Sesardic. Tel: 01707 654753; Fax: 01707 646730; Email: dsesardic@nibsc.ac.uk

A number of *in vitro* alternative assays have been described for the detection of toxin, mostly based on immunodetection. Some of these assays were less sensitive than the mouse bioassay such as reports of Smith, (1977), Notermans *et al.* (1978), Sugiyama, (1980) and Ashton *et al.* (1985), while others suffered from cross-reactivity between the different serotypes of toxin as reported by Dezfulian and Bartlett (1984) and Thomas (1991). Recent improvements in enzyme-immunoassays for BoNT/A have attempted to address these issues by the use of very sensitive and specific reagents, such as monoclonal antibodies, as well as technically complicated signal amplification systems as reported by Gibson *et al.* (1988), Doellgast *et al.* (1993) and Kumar *et al.* (1994). Although these assays have been able to achieve levels of specificity and sensitivity for toxin detection equal to or better than the mouse bioassay, their general usefulness has been limited because of the complexity of the necessery detection systems. More importantly, these assays lacked correlation with the *in vivo* mouse bioassay, because of the general inability of ELISAs to distinguish between active and inactive toxin (Ekong *et al.*, 1995, Sesardic, 1996).

BoNT/A is now known to be a zinc-dependent endopeptidase whose toxic action depends on the specific cleavage of a synaptic protein SNAP-25 (synaptosomal associated protein of molecular weight 25 kD). In this presentation, a method for the measurement of toxin activity is described, based on the intracellular mode of action of the toxin, which involves measurement of the proteolytic cleavage of recombinant fragment of SNAP-25 by BoNT/A.

Materials and Methods

Preparation of recombinant fragment of SNAP-25.

The nucleotide sequence coding for a fragment of SNAP-25 spanning the toxin cleavage site (residues 134–206) was amplified by PCR and inserted into the pMAL-c2 expression vector such that it was in frame with *malE* of *E.coli*. The vector had the factor Xa recognition site to facilitate subsequent purification of the SNAP-25 fragment. Expression was controlled by the strong inducible P_{tac} promoter. Transformed *E.coli* cells (containing SNAP-25-fusion/pMAL-c2 plasmids) were grown at 37 °C and expression of fusion protein was induced by the addition of n-isopropylthio-β-D-galactoside. Bacteria were harvested and cells lysed by osmotic shock. Supernatant fluids containing the MBP-SNAP-25 fusion protein were purified by affinity chromatography on a cross-linked amylose column. Fractions containing fusion protein were pooled and cleaved by factor Xa to release the recombinant fragment.

Production and characterization of anti-SNAP-25 antibodies

Two C-terminally targeted antibodies (R1 and R2) specific for SNAP-25 fragments (Fig. 1A) were prepared in rabbits and used to monitor cleavage of the recombinant substrate by BoNT/A. Synthetic peptides of SNAP-25 corresponding to residues 193–206 (R1) and 190–197 (R2) were coupled to Keyhole Limpet Haemocyanin (KLH) and used for immunization. Animals were injected with four doses of each immunogen containing 300 μg. Purified immunoglobulin was prepared by ammonium sulphate precipitation of immune serum followed by Protein G affinity chromatography. Antibodies were screened for specificity by ELISA using peptide-coated microtitre plates and goat-anti rabbit IgG conjugated to HRP.

Microtitre-based endopeptidase assay for BoNT/A activity

A microtitre-based assay for the estimation of BoNT/A activity in clinical preparations was developed using the recombinant SNAP-25 substrate and anti-peptide antibodies described in Fig. 1A. Microtitre plates were coated with 0.1–0.2 μg/well of substrate and treated with serial dilutions of previously reduced clinical preparations of BoNT/A at 37 °C for 1h. The amounts of uncleaved substrate and cleavage product were quantified using the R1 and R2 antibodies and goat anti-rabbit IgG conjugated to HRP. The assay was optimized by examining the influence of a number different conditions on the cleavage reaction. There was an absolute requirement for toxin to be pre-reduced, and this was achieved by incubation for 30 min at 37°C with 10 mM DTT in the reaction buffer. DTT had a biphasic effect on toxin activity and was inhibitory at >20 mM concentration. The cleavage reaction had a pH optimum at pH 7.0, and was strongly inhibited by the presence of NaCl. HEPES buffers gave the best rates of cleavage at all the concentrations examined, and the optimized assay employed HEPES buffer at a concentration of 50 mM.

The optimized assay was used to determine the relative proteolytic activities of different clinical formulations and these values were compared with relative potency estimates obtained with the conventional mouse bioassay.

Bioassay

The potency estimates of the clinical preparations of BoNT/A were performed using the mouse bioassay as previously described (Sesardic *et al.*, 1996).

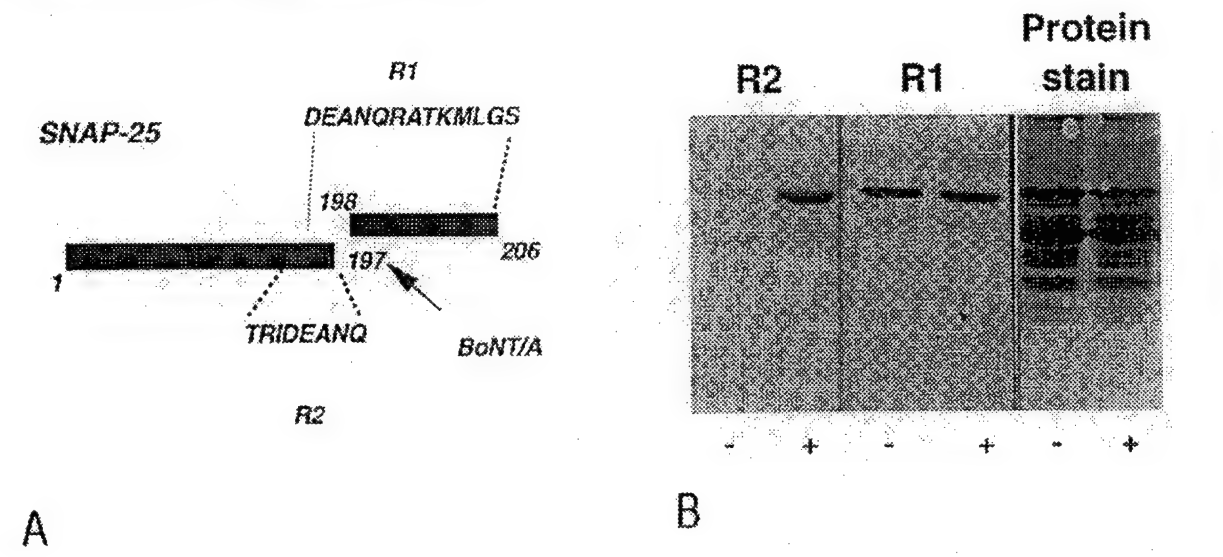


Figure 1. *Specificity of targeted anti-SNAP-25 antibodies.* A. Schematic representation of SNAP-25 indicating specificities of targeted antibodies; R1 antibody (residues 193–206) and R2 antibody (residues 190–197) as antigen. B. SDS-PAGE and Western blotting of recombinant fragment of SNAP-25 before (–) and after (+) treatment with BoNT/A. Following electrophoretic transfer to nitrocellulose membranes, blots were stained for total protein (protein stain) or with the R1 or R2 antibodies as indicated.

Results

Targetted anti-SNAP-25 antibodies

Two targetted anti-SNAP-25 antibodies (R1 and R2) were prepared in rabbits. Antibody specificities and titres were determined by ELISA and Western blotting. Antibodies were specific for their respective peptide antigens with no cross-reactivity to other SNAP-25 peptides and were high titre (0.08 μg/ml for R1 and 1.1 μg/ml for R2 antibody). Western blotting confirmed that the R1 antibody recognised SNAP-25 and that the R2 antibody was specific for the BoNT/A cleaved fragment of SNAP-25 (Fig. 1B).

Preparation of recombinant fragment of SNAP-25 and cleavage by BoNT/A

Extracts from induced *E. coli* cultures that had been transformed showed the presence of a fusion protein of the expected molecular weight (50 kD), consistent with fusion of maltose-binding protein and the SNAP-25 fragment (42kD and 7kD respectively). This protein was purified in one step on a cross-linked amylose column and the recombinant fragment was obtained after cleavage with factor Xa. Both the fusion protein and the recombinant fragment were recognized by the R1 antibody, specific for the C-terminus of SNAP-25. Purified fusion protein and recombinant fragment of SNAP-25 were efficiently cleaved by either purified or clinical BoNT/A, demonstrated by the gain of reactivity to the R2 antibody (Fig. 1B).

Estimation of acitvity of BoNT/A in clinical formulations

The endopeptidase assay was used to examine a single preparation of therapeutic toxin to determine the sensitivity which was estimated as 0.2–0.8 MLD_{50}/ml (n = 6). The titration curve showed a linear response between 0.5–63 MLD_{50}/ml. The assay showed good reproducibility, with inter and intra-assay variability of 5.9 to 11.2% gcv.

Relative potencies of different therapeutic preparations of BoNT/A from different manufacturers were compared by the *in vitro* endopeptidase and mouse bioassay. There was a very good correlation between the two assays for the preparations examined with the correlation coefficient of $r = 0.93$ ($n = 31$), and slope of 1.03 ± 0.02 showing that the mean difference between the potency estimates does not significantly differ from 0 (paired t-test $P > 0.5$). Thus the amount of biologically active BoNT/A detected in

Sesardic D (1996) Requirements for valid alternative assays for testing of biological therapeutic agents *Devel Biol Standard* **86**, 311–318.

Sesardic D, McLellan K, Ekong TAN, Gaines Das R (1996) Refinement and validation of an alternative bioassay for potency testing of therapeutic botulinum type A toxin *Pharmacol Toxicol* **78**, 283–288.

Smith LDS (1977) In Charles C Thomas (ed): *Botulism; the organisms, its toxins, the disease*. Springfield: Illinois; pp 75–77.

Sugiyama H (1980) *Clostridium botulinum* neurotoxin. *Microbiol Rev* **44**, 419–448.

Thomas RJ (1991) Detection of *Clostridium botulinum* types C and D toxin by ELISA *Australian Vet J* **68**, 111–113.

Posters

Measurement of a synaptobrevin-thioredoxin fusion protein (VAMP II 51aa-T) by capillary zone electrophoresis using laser induced fluorescence detection.

Asermely, K. E., Nowakowski, J., Courtney, B. and Adler, M.

Neurotoxicology Branch, USAMRICD, Aberdeen Proving Ground, MD 21010. USA.

Capillary Zone electrophoresis (CZE) was utilised to measure a 51 amino acid Asp (43)-Lys(94) peptide fragment of a human neuronal protein, synaptobrevin II or Vesicle Associated Membrane Protein II (VAMP II). The synaptobrevin peptide fragment (6 kDa) was expressed as an 18 kDa fusion protein with thioredoxin (12 kDa). The thioredoxin fusion to the synaptobrevin peptide fragment allows rapid isolation at high purity from E. coli. The synaptobrevin-thioredoxin fusion protein was derivatized using a fluoregenic reagent, 3-(4-carboxybenzoyl)-2-quinoline-carboxaldehyde (CBQCA), and measured by laser induced fluorescence (LIF). For derivatization, the synaptobrevin-thioredoxin fusion protein in osmotic shock buffer, pH 8.0 was incubated with CBQCA in the presence of KCN for 1 hr at room temperature. The derivatized protein was separated in the CZE through an open silica uncoated capillary in sodium borate buffer at pH9.0. Glycine was derivatized under similar conditions and used as a marker for the experiment. The synaptobrevin peptide fragment migration time was 7.8 minutes under the above conditions. Experiments were done to optimise the conditions for measurement of the synaptobrevin-thioredoxin fusion protein in CZE. These preliminary results suggest that Capillary Zone Electrophoresis (CZE) may be useful analytical technique to study peptide fragments of neuronal proteins which are substrates for botulinum toxins.

Studies on the cluster of genes encoding the botulinum neurotoxin complex in *Clostridium botulinum* strains of different antigenic toxin types.

East, A. K., Bhandari, M., Hielm, S., Collins, M. D.

Microbiology Department, Institute of Food Research, Early Gate, Whiteknights Road, Reading, RG6 6BZ.

Genes encoding the components of the botulinum neurotoxin (BoNT) complex, consisting of the toxin and one or more non-toxic proteins, are clustered in strains of clostridia which produce BoNT. The size of the complex differs from strain to strain and seems to be dependent on the antigenic type of the toxin produced. The smallest complex, found in all BoNT-producing strains regardless of antigenic type, the M-complex (rmm 300k) consists of BoNT and another protein of approximately the same size, the nontoxic-nonhemagglutinin component (NTNH). The gene encoding NTNH is found immediately upstream of BoNT. Larger complexes contain components with hemagglutinin activity and the genes encoding these are also clustered with that of BoNT. In addition to the composition of the BoNT complex varying with toxin type, the genes encoding the complex components and their organisation also differs. The inter-relationship of the genes in this linked cluster is discussed.

Chimeric toxins between botulinum neurotoxin light chain or C_3 enzyme, and iota toxin.

Marvaud, J. C. and Popoff, M. R.

Unité des Toxines Microbiennes, Institute Pasteur, 28 rue de Dr. Roux, 75724 PARIS, Cedex 15, France.

Clostridial binary toxins such as *Clostridium botulinum* C_2 toxin, *Cl. perfringens* iota toxin and *Cl. spiroforme* toxin, consist of two separate protein chains non-linked neither by a disulfide bridge nor covalent bond. The enzymatic component (Ia of iota toxin) catalyses the ADP-ribosylation of cellular actin, and the binding component (Ib of iota toxin) recognises a cell surface receptor and allows the internalisation of Ia into the cytosol. The iota toxin which is active on numerous cell lines, was used to internalise heterologous proteins into cells. The C_3 enzyme from *Cl. botulinum* was selected to investigate this model. In order to determine the Ib-binding domain of Ia, different parts of Ia were fused with the C_3 enzyme. The purified chimeric proteins were tested in presence of Ib, for their ability to depolymerise the actin cytoskeleton of Vero cells. We found a Ia fragment which is able to translocate efficiently the C_3 enzyme in presence of the Ib component. A similar construction was done with the C_1 light chain instead of C_3 enzyme, in order to investigate the role of syntaxin in exocytosis in various cells.

Investigation of a strain of *Clostridium botulinum* producing more than one type of botulinum neurotoxin.

Santos-Buelga, J., Collins, M. D. , East, A. K.

Microbiology Department, Institute of Food Research, Early Gate, Whiteknights Road, Reading. RG6 6BZ.

Strains of the species *Clostridium botulinum* produce the extremely potent botulinum neurotoxin (BoNT) the causative agent of botulism. BoNTs have been classified serologically into seven antigenic types (A to G). Most strains produce toxin of one antigenic type only, but some produce BoNTs of more than one type. Recent evidence has shown that almost half of proteolytic *Cl. botulinum* type A strains have a type B BoNT gene present (1). In most of these strains the type B gene is not expressed (and therefore described as 'silent') but in other strains, so called 'dual' toxin strains, more than one type of BoNT is produced. Recent studies have investigated the BoNT genes of dual toxin producing strains of type AB (2) and in an A(B) strain (type A with a silent type B gene) the organisation of the two gene clusters was investigated (3). Results of work on another dual toxin producing strain of *Cl. botulinum* are presented and discussed.

References

(1) Franciosa et al. 1994, J. Clin. Microbiol. 32, 1911-1917
(2) Fujinaga et al. 1995, BBRC 213, 737-745
(3) Hutson et al. J. Biol. Chem. In press

Quantitative assessment of the effect of heat treatment and subsequent incubation temperature on growth from spores of non-proteolytic strains of Clostridium botulinum.

Stringer, S. C., Fairburn, D. A. and Peck, M. W.

Institute of Food Research, Norwich Research Park, Colney, Norwich. NR4 7UA.

The microbiological safety of sous-vide foods and other refrigerated processed food extended durability (REPFEDs) relies on mild heat treatment followed by storage at refrigeration temperatures. non-proteolytic strains of *Clostridium botulinum* are a safety concern in these products, as they form endospores that survive mild heat treatment and are able to grow and produce a powerful neurotoxin at a temperature as low as 3.3°C. The continued safe development of these minimally processed foods requires knowledge of the ability of spores on non-proteolytic *Cl. botulinum* to withstand heat treatments and to germinate, grow and produce toxin at refrigeration temperatures. In the work reported here, we have quantified the effect of heat treatment combined with subsequent incubation at refrigeration temperatures on growth and toxin production from spores of non-proteolytic *Cl. botulinum*.

Spores of six strains of non-proteolytic *Cl. botulinum* (two each of type B, E and F) were heated at 90°C for between 0 and 60 min and subsequently incubated at 5°C, 10°C or 30°C in broth containing lysozyme. The most probable number of spores leading to turbidity depended on the combined heat treatment, incubation time and incubation temperature. Heating at 90°C for one or more min ensured a 10^6 reduction when spores were subsequently incubated at 5°C for up to 23 weeks. At 10°C, heating at 90°C for 60 min ensured a 10^6 reduction over 23 weeks. The same treatment did not reduce the spore population by 10^6 when they were subsequently incubated at 30°C for four days. Incubation at 5°C reduced the number of spores leading to turbidity by 1000-fold compared with 30°C, whilst incubation at 10°C reduced it 10-fold. These proportions were not affected by prior heating of the spores at 90°C for up to 60 min.

Neurophysiological investigation of patients with botulism.

Critchley, E. M. R.

Consultant Neurophysiologist, Royal Preston Hospital, Sharoe Green Lane North, Fulwood, Preston, LANCS. PR2 9HT.

This Poster emphasises that in the first patients who became severely flaccid in all four limbs within a short space of time conventional electrophysiological tests showed conduction blocks and were unhelpful In establishing the diagnosis. As soon as the milder affected patients were examined it became clear that many of these had the classical post-tetanic facilitation from which the diagnosis of a defect at the neuromuscular junction can be made.

Long term follow up of botulism patients.

Critchley, E. M. R.

Consultant Neurophysiologist, Royal Preston Hospital, Sharoe Green Lane North, Fulwood, Preston, LANCS. PR2 9HT.

A selection of patients from the middle age group, that is to say excluding the elderly and paediatric patients, were examined two years or more after the outbreak of acute botulism. Many still complained of fatigue-like symptoms and had not returned to their previous state of health. This seemed not to correlate with the severity of their initial illness. This finding which has been previously reported in other outbreaks was examined without finding the cause. Unfortunately there is a limit to the type of testing which was possible and one cannot exclude a defect in the mitochondria.

Characterisation of haemagglutinins composing *Clostridium botulinum* progenitor toxins.

Inoue, K., Fujinaga, Y. Watanabe, T., Takeshi, K., Ohyama, T., Kozaki, S., Inoue, K. and Oguma, K.

Dept. of Bacteriology, Okayama University Medical School, 2-5-1 Shikata-cho, Okayama 700. Japan.

Clostridium botulinum strains produce seven immunologically distinct neurotoxins. These neurotoxins associate with non-toxic components to become large complexes (progenitor toxins) in the culture supernatants. Type A strain produces three different-sized progenitor toxins with Mr of 900 k (19 S), 500 k (16 S), and 300 k (12S). type B, C, and D strains produce the 16 S and 12 S toxins. In all of these toxin types, the non-toxic components of the 19 S and 16 S toxins have hemagglutinin (HA) activity, but that of the 12 S toxin does not. Therefore, it was postulated that the 12 S toxin consists of a neurotoxin and a non-toxic component having no hemagglutinin activity (non-toxic-nonHA), and the 19 S and 16 S toxin contains hemagglutinin (HA) component in addition to the 12 S toxin. The progenitor toxins having HA activity (19 S and 16 S toxins) were purified from type A, B, C, and D strains, and their HA activities were characterised.

Type A 19 S and 16 S toxins, and type C and D 16 S toxins showed HA activity to human erythrocytes but type B 16 S toxin showed little activity. When the erythrocytes previously treated with neuraminidase were employed, HA titres of type C and D 16 S toxins decreased, but that of type B 16 S toxin increased. HA titres of type A 19S and 16 S toxins were little changed whether neuraminidase treated or nontreated erythrocytes were used. These results suggest that HAs of different type progenitor toxins bind to different polysaccharides on the surface or erythrocytes.

Blockade of ACh release at a synapse in *Aplysia* by a peptide that mimics the carboxy-terminal domain of SNAP-25.

Apland, J. P., Filbert, M. G., Adler, M., Ferrer-Montiel, A. V., and Montal, M.

Dr. J. P. Apland, Neurotoxicology Branch, USAMRICD, Aberdeen Proving Ground, MD 21010. USA.

Neurotransmitter exocytosis is preceded by docking of synaptic vesicles at active sites on the presynaptic membrane. SNAP-25 (synaptosomal-associated membrane protein of 25 kDa) is one of several proteins forming the fusion complex at the active sites. Botulinum neurotoxins A and E block neurotransmitter release by cleaving SNAP-25. The SNAP-25 C-terminal 20-mer peptide (named ESUP, for excitation-secretion uncoupling peptide) was recently shown by Gutierrex *et al.* (FEBS Lett. 372:39) to inhibit transmitter release from permeabilized bovine chromaffin cells. The effect of this peptide on ACh release at an identified cholinergic synapse of Aplysia neurons was investigated in preliminary experiments in our laboratory.

Recordings were obtained from isolated buccal ganglia of Aplysia. The presynaptic neuron was current-clamped and stimulated electrically at 0.1 Hz to elicit action potentials. The postsynaptic neuron was voltage-clamped, and evoked inhibitory postsynaptic currents (IPSPs) were recorded. ESUP was pressure-injected into the presynaptic neuron, and its effect on the amplitude of the IPSPs was studied. ACh release from presynaptic cells, as measured by the amplitudes of IPSPs, was consistently inhibited. The inhibition was gradual, requiring 2-3 hr to effect a 50-60% reduction of IPSP amplitude. A random-sequence peptide of the same amino acid composition had no effect. These results suggest that ESUP competes with the intact SNAP-25 for binding with other fusion proteins, thus inhibiting exocytosis of neurotransmitter. This effect may account, in part, for botulinum toxin-induced inhibition of transmitter release.

Genetic analysis of neurotoxin genes in the type A Clostridium botulinum strain NCTC 2916.

Henderson, I., Davis, T. O., Whelan, S. M., Elmore, M. E. and Minton, N. P.

Research Division, Molecular Microbiology Dept., CAMR, Porton Down, Salisbury. Wilts. SP4 0JG.

The botulinum neurotoxin (BoNT) protein serotypes (A-G) are expressed in association with other "non-toxic" proteins that enhance toxin potency. The form and size of the complex can include haemagglutinating proteins (HA), as well as a non-toxic, non-haemagglutinating protein (NTNH), and is serotype dependent, ranging from the M form (300 kDa, all serotypes -no haemagglutinating activity), to the L form (500 kDa, serotypes A, B, C, D and G) and finally to the LL form (900 kDa, serotype A). A genetic analysis of the botA gene locus in the type A *Clostridium botulinum* strain NCTC 2916 has shown that the genes of the toxin complex form two divergent operons, comprising [HA34, HA17, HA70] and [NTNH/A, botA], encoded on opposite strands. These operons have conserved promoter sequences and are separated by an open reading frame encoding a putative DNA binding protein (OrfX) with features in common with other transcriptional regulators. This suggests that toxin complex gene expression is regulated by a co-ordinated transcriptional mechanism. An analysis of NCTC 2916 using neurotoxin gene specific oligonucleotides by PCR has also detected a botB gene. An investigation of this gene has shown that it possesses a translational stop codon (GAG→TAG). This supported the finding that no BoNT/B expression could be detected from this strain and that botB was therefore silent. Further analysis of the region upstream of botB revealed the presence of a functional gene comprising parts of both the NTNH/A (5') gene and a NTNH/B (3') gene. This hybrid is preceded by sequence identical to that found upstream of the NTNH/A gene at the botA locus, suggesting that the botB locus arose by recombination.

Evidences for a participation of TGAse II/Gh in the intracellular mode of action of tetanus neurotoxin.

Deloye, F., Poulain, B., Luini, A. and Facchiano, F.

LNBCM, CNRS, F-91198, Gif-sur-Yvette, France.

Recent evidences indicate that VAMP/synaptobrevin cleavage, while certainly involved, cannot alone satisfactorily explain all the effects of tetanus neurotoxin (TeNT) on neurotransmission. Indeed, TeNT light chains mutated in the catalytic site, while completely devoid of protease activity against VAMP/synaptobrevin, are able to inhibit neurotransmission when intracellularly applied (Ashton et al., 1995). Therefore, TeNT might non-proteolytically act on other intracellular targets involved in controlling neurosecretion. We have previously hypothesised that the enzyme tissue transglutaminase type II (TGase IIGαh) might be a target for TeNT (Facchiano F. and Luini A. 1992). We have examined whether TGase IIGαh plays a role in the neurosecretion-blocking action of TeNT at rat brain synaptosomes releasing glutamate and at cholinergic synapses in *Aplysia californica*. We find that TeNT markedly activates the intrasynaptosomal TGase *in vivo*, with a potency and time course parallel to those with which it inhibits neurotransmitter release. Moreover, the intraneuronal application of specific TGase blockers, including anti-TGase antibodies, synthetic TGase inhibitors and a TGase synthetic substrate peptide markedly inhibit the TeNT-induced neurosecretory block. These, together with previously published findings demonstrate a role for TGase IIGαh in the mechanisms of action of TeNT and imply and physiological role of TGase in the exocytotic process.

Ashton, A. C., Li, Y., Doussau F., Weller, U., Dougan G., Poulain, B & Dolly, J. O. (1995). J. Biol. Chem. 270, 31386-31390.
Facchiano F. and Luini A. (1992) J. Biol. Chem. 267, 13267-1371

Effectiveness of aminoquinolines against Type A botulinum neurotoxin-induced paralysis in mouse skeletal muscle.

Deshpande, S. S., Sheridan, R. E. and Adler, M.

Neurotoxicology Branch, USAMRICD, Aberdeen Proving Ground, MD 21010. USA.

4- and 8-amino substituted quinolines and quinacrine which are effective antimalarial agents, were tested as potential pre-treatment compounds for antagonising block of nerve-elicited muscle contractions in diaphragms exposed to botulinum type A neurotoxin (BoTx-A). 4-aminoquinolines (chloroquine, amodiaquine) and quinacrine prolonged BoTx-A induced neuromuscular block by more than three-fold of control (BoTx-A alone). 8-aminoquinolines primaquine and WR242511 (8-14-amino-1-methylbutyl] amino-2-methoxy-5-hexoxy quinoline) had no anti-BoTx activity.

Rank order potency for the test compounds was - amodiaquine > quinacrine > chloroquine > quinine or quinidine. Antimalarial drug pyrimethamine which lacks quinoline ring structure was ineffective. Maximum protection from BoTx-induced neuromuscular block was achieved when the muscles were exposed to the drug prior to or at the same time with the toxin. All the test compounds except quinine and quinidine inhibited muscle contractility at concentrations greater than 20 μM. Amodiaquine (50 μM) also inhibited contraction of the diaphragms. Combinations of agents that are "universal antagonists" and act at different steps of BoTx-A poisoning (i.e. binding, internalisation and proteolytic activity) delayed the time to 50% block up to the maximum produced by the most effective single agent. Low doses of lectin from *Triticum vulgaris*, TPEN (N, N, N', N'-tetrakis(2-hydroxypropyl) ethylenediamine) and quinacrine in combination produced an additive effect (approximately 4-fold increase in time to 50% block over control). Similar results could also be obtained by using higher concentrations of TPEN or quinacrine alone. However, these concentrations led to neuromuscular or cellular toxicity. In conclusion, 4-aminoquinolines are potent antagonists of botulinum neurotoxins when administered prior to or with BoTx-A. The mechanism of protection is, in all likelihood, through raising endosomal pH above 5.5, a value critical for the release of toxic light chains into the nerve terminal cytoplasm. Some of these drugs could also act by inhibiting channel formation. The inherent toxicity of these drugs precludes their practical use against botulinum poisoning at the present time.

Translocation of tetanus toxin: studies in two different in vitro systems.

Lücke, T., Göschel, H. and Bigalke, H.

Institute of Toxicology, Hannover Medical School, 30623 Hannover, Germany.

Tetanus Toxin (TeTx) is a dichain protein (MR 50.000) that blocks the exocytotic release of transmitters. A disulphide bond and ionic interactions link its two components. (1) The heavy chain (MR 100,000) navigates the holotoxin from the extracellular space into the cytosol. (2) The light chain (MR 50,000) is a highly specialised Zn^{2+}-dependent metallo-protease specifically proteolysing the SNARE-protein synaptobrevin II, thereby inhibiting exocytosis. At physiological pH, TeTx is taken up via receptor-mediated endocytosis. It was postulated that acidification of the endosome causes conformational changes necessary for the translocation of TeTx from the endosome into the cytosol. Since, at low pH, TeTx forms pores in neuronal membranes, the pore-forming activity might also be involved in the translocation of the toxin. In spinal cord neuronal networks TeTx (100 ng/ml) induces - within a latent period of 5 hours - bursting activity similar to that provoked by strychnine (10 µM). The bursts were prevented when cells were simultaneously exposed to the vacuolar H^+-ATPase inhibitor bafilomycin A1 (10 nM). However, they appeared one hour after the exchange of TeTx and bafilomycin A1 for TeTx antibodies. Once established, TeTx-induced bursts were resistant to bafilomycin as those elicited by strychnine. In addition to central effects TeTx (10 µg/ml) caused paralysis of the mouse diaphragm in vitro both at pH 5 and pH 7.4. Bafilomycin delayed paralysis at pH 7.4 only. Bafilomycin A1 is a powerful agent to prevent, at physiological pH, the action of TeTx in spinal cord neurons as well as in motor nerve endings. Thus at pH 7.4 the acidification of the endosomal compartment seems to be essential for the translocation of TeTx into the cytosol. Since the inhibition of acidification did not prevent the action of TeTx at pH 5, it must be translocated directly from the extracellular space into the cytosol. The artificial extracellular environment probably reflects the acid conditions within the endosome.

Structural determinants of the specificity for VAMP/ Synaptobrevin of tetanus and botulinum type B and G neurotoxins.

Pellizzari, R., Rossetto, O., Lozzi, L., Giovedi, S., Johnson, E., Shone, C.C. and Montecucco, C.

Dept. of Biomedical Science, University of Padova, Via Trieste 75, 35121 Padova, Italy.

Tetanus and botulinum neurotoxins type B and G are zinc endo-peptidases of remarkable specificity. They recognise and cleave VAMP/synaptobrevin, an essential protein component of the vesicle docking and fusion apparatus. Some evidence indicates that the cleaved region is not the sole determinant of the specific recognition of tetanus and botulinum neurotoxins. In addition to the sequence flanking the peptide bond to be cleaved, we have identified a nine residue motif present in all SNAREs. This SNARE motif is included within regions of the three SNAREs predicted to adopt an alpha-helical conformation and is characterised by the presence of three negatively charged residues spaced in such a way that the Edmundson wheel plot shows a negatively charged surface. VAMP contains two copies of this motif, named V1 and V2. Antibodies raised against this motif cross-react among VAMP, SNAP-25 and syntaxin and inhibit the VAMP-2 proteolytic cleavage by tetanus and botulinum neurotoxins. Moreover, in the same conditions, SNAP-25 and syntaxin specific neurotoxins inhibit tetanus and botulinum neurotoxin proteolysis of VAMP, confirming the idea that each clostridial neurotoxin should be able to bind any SNARE protein. The role of the three negatively charged residues of the motif in neurotoxin recognition of VAMP-2 was probed by site-directed mutagenesis. Progressive substitutions of acidic residues in the V2 motif affect protein cleavage by BoNT/B and /G, but unexpectedly TeNT cleaves these VAMP mutants at similar rates as the control. On the contrary, mutations of the negatively charged residues of V1 motif of VAMP affect protein cleavage by TeNT, but not by BoNT/B and /G. Though the L chains of TeNT and BoNT/B are predicted to be very similar, these results suggest that there is a subtle difference in the way these toxins recognise their substrate.

Tissue and cell distribution of VAMP/synaptobrevin isoforms.

Rossetto, O., Gorza, L., Schiavo, G., Schiavo, N., Scheller, R. H. and Montecucco, C.

Dept. of Biomedical Science, University of Padova, Via Trieste 75, 35121 Padova, Italy.

VAMP/synaptobrevin, a major component of the synaptic vesicle membrane, plays a central role in neuroexocytosis since it is the specific target of the zinc-endopeptidase activity of tetanus and botulinum B, D, F and G neurotoxins, which cause a sustained inhibition of neurotransmitter release. Two VAMP isoforms are expressed in the nervous and neuroendocrine system and are differently distributed among the various specialised parts of the tissue.

We studied the distribution of neuronal isoforms VAMP-1 and VAMP-2 mRNA outside the nervous tissue by in situ hybridisation experiments and by RNase protection assay using c-RNA anti-sense probes. VAMP proteins were then detected via western blots of tissue homogenates and light and confocal immunofluorescence microscopy with isoform specific antibodies raised against the amino-terminal portion of VAMP, the part which differs among the two isoforms.

Here we show that the VAMP proteins are highly expressed in tissues with a high secretory activity such as thyroid and pancreas, and, in lower proportions, in all tissues tested including liver, heart atrium and smooth muscle cells. The two isoforms are differentially expressed in various tissues and in some specialised areas their presence is mutually exclusive. The most striking case is that of pancreas with VAMP-1 protein present in the exocrine acinic cells and VAMP-2 being predominantly expressed in the endocrine Langerhans islets. Intracellular distribution in undifferentiated PC12 cells shows a spotty presence of VAMP-2 within the cytosol. Neuronal differentiation of these cells is accompanied by a large increase in the amount of VAMP-1 and in the appearance of intense staining of both VAMP-1 and -2 in synaptic terminals.

The present results provide evidence for the importance of neuronal VAMP proteins in the physiology of all cells and suggest that the two isoforms would have specialised functions.

Botulinum neurotoxin C acts on syntaxin and SNAP-25 and is cytotoxic to neurons.

Williamson, L. C., Halpern, J. and Neale, E. A.

Laboratory of Developmental Neurobiology, NICHD, NIH, Building 49, Room 5A38, Bethesda, MD 20892. USA.

Botulinum neurotoxins (BoNTs) block neurotransmitter release by cleaving specific synaptic proteins necessary for vesicle docking and/or fusion. We have compared the actions of BoNTs A, B and C on their respective substrates SNAP-25, VAMP and syntaxin, in developing and mature spinal cord neurons in cell culture. We demonstrate that BoNT C, in intact neurons, cleaves not only syntaxin, but also exerts a secondary effect on the carboxyl terminus of SNAP-25. Furthermore, BoNT C (0.3 nM), unlike BoNTs A or B, is severely cytotoxic for both young and mature neurons. In newly-plated spinal cord cultures, neurons do not survive beyond five days following treatment with BoNT C. Neurite outgrowth and synapse formation are markedly inhibited by BoNT C. We do not observe any effect on neurite outgrowth or synaptogenesis when VAMP is cleaved by BoNT B or when SNAP-25 is cleaved by BoNT A. In mature cultures, synaptic terminals become enlarged within 24 hours of BoNT C exposure. During the next four days, axons degenerate and dendrites and cell bodies are lost. Electron microscopy after two days of BoNT C exposure reveals synaptic terminals packed with vesicular elements (100-200 mm) and membranous dense bodies indicative of degeneration. Synaptic release sites remain relatively intact with synaptic vesicles accumulated at most active zones. This cytotoxicity is unique to BoNT C and is specific to neurons; underlying non-neuronal cells are unaffected. BoNTs A, B and C each block neurotransmitter release in these cultures at the concentration studied, but only BoNT C causes overt neuronal degeneration. These observations suggest that syntaxin, alone or in combination with SNAP-25, may be necessary for neuronal survival. The cytotoxicity of BoNT C may be significant in terms of its efficacy for the clinical treatment of muscle spasm disorders.

Regulated secretion in BoNT/A L-chain stably transfected AtT 20 cells.

[1]Aguado, F., [2]Gombau, L., [2,3]Blanco, J., [1]Marsal, J. and [1]Blasi, J.

Universitat de Barcelona, Dept. de Biologia Cellular i Anantomia Patològica, Divisio de Ciències de la Salut, C/Casanova 143. 08036 Barcelona. SPAIN.

Neurons and endocrine cells secrete chemical signals upon appropriate stimulation. Synaptic proteins such as synaptobrevin, syntaxin and SNAP-25 have been identified in several hormone secretory cell types. These proteins are constituents of the protein complex involved in regulated exocytosis and are the substrates for clostridial neurotoxins (CTx). Light chains of CTx need to gain access to nerve terminal to exert their proteolytic activity on these proteins. This is achieved by specific binding of the toxin molecule through its heavy chain to nerve terminal surface receptors followed by endocytosis, sorting and translocation of the light chain. Endocrine cells lack CTx receptors on their surface and light chains must be introduced into the cell by other means. In this study, the ACTH secretory cell line (ATt20) was transfected with a plasmid that confers neomycin resistance and contains the coding sequence for the BoNT/A light chain with protein expression controlled by the cytomegalovirus promoter. Neomycin resistant clones were further screened for SNAP-25 cleavage. Stably transfected clones with cleaved SNAP-25 were obtained. These cells showed no morphological changes when compared to controls. In particular cytoskeleton and granule patterns were not affected. ACTH release induced by 8-Br-cAMP and latrotoxin was impaired in cells that express L-BoNT/A. These results suggest a direct involvement of SNAP-25 in the hormone secretion mechanism. Furthermore, this shows that hormone secretory cell lines can be used to further increase our knowledge both on CTx blockade mechanism of exocytosis and on the molecular machinery involved in hormone secretion using CTx as molecular tools.

Primary embryonic rat dorsal root ganglia cultures: characterisation and susceptibility to *Clostridium botulinum* neurotoxins.

Welch, M. J., Duggan, M. J. and Foster, K. A.

Research Division, CAMR, Porton Down. Salisbury. Wilts. SP4 0JG.

Dorsal root ganglion (DRG) cultures were prepared from embryonic rats (15 days gestation), by dissociation with trypsin/EDTA, and maintained in medium for up to four weeks. These DRG cultures were shown to be predominantly neuronal by using antibodies to a variety of standard cellular markers such as neurofilament and glial fibrillar acidic protein.

The DRG cultures were also shown to possess the known *Cl. botulinum* neurotoxin substrates SNAP25, syntaxin and synaptobrevin, and the cultures were demonstrated to be sensitive to exogenously added neurotoxin. The characteristics of the effects of neurotoxin on the DRG cultures were examined.

Role of zinc in the polypeptide folding of botulinum neurotoxin.

Fu, F. N. and Singh, B. R.

Dept. of Chemistry and Biochemistry, UMass, Dartmouth, 285 Old Westport Road, North Dartmouth. MA 02747 USA.

Botulinum neurotoxins are a new group of zinc endopeptidases which cleave different proteins of the SNARE complex, and block the neurotransmitter release. The short zinc binding motif, HExxH, located in the middle of the light chain sequence is responsible for the enzymatic activity of the neurotoxin. it has been speculated that zinc may play a structural role in addition to its catalytic role.

FT-IR spectroscopic analysis revealed an increase (13%) in α-helical structure upon removal of the zinc from the neurotoxin. Replenishment of the zinc partially restored the secondary structure. However, excess amount of zinc tended to disrupt the structure again. Solute quenching technique was applied to investigate changes in the micro-environment of tryptophan residues. Higher quenching constants were observed for the acrylamide quenching of the neurotoxin after zinc-removal. Near UV CD spectral analysis indicated change in the topography of Trp as reflected by the disappearance of the band at 295 mm upon removal of the zinc. In a further effort to evaluate the role of zinc in the polypeptide folding of the neurotoxin, its thermal stability was analysed in the absence and presence zinc. With the bound zinc, the neurotoxin exhibited a normal two-state unfolding with DH of 513.8 kJ/mole and DS of 1.58 kJmol-1S-1 at Tm (52.5°C). Removal of zinc precluded a transition up to 100°C, suggesting a strong role of Zn^{2+} in the maintenance of the neurotoxin tertiary structure.

Delivery of recombinant tetanus toxin-superoxide dismutase proteins to central nervous system neurons by retrograde transport.

Figueiredo, D. M., Hallewell, R. A., Chen, L. L, , Dougan, G., Savitt, J., Fishman, P. S. and Fairweather, N. F.

Department of Biochemistry, Imperial College of Science, Technology & Medicine, South Kensington, London SW7 2AZ

Tetanus toxin is delivered very efficiently to the CNS by retrograde axonal transport. The 50 kDa non-toxic fragment C to tetanus toxin (TTC) contains ganglioside and neuronal cell binding activities and undergoes retrograde transport in the absence of the remaining fragment of the toxin. We are interested in transport of CuZn superoxide dismutase (SOD) to the spinal cord, because increased levels of SOD are protective in experimental animal models of stroke and Parkinson's disease, whereas mutations in SOD cause motor neuron disease.

Using an *E. coli* expression system and maltose binding protein (MBP) as an aid to purification SOD/TTC fusion proteins were expressed in two orientations: MBP-SOD-TTC and MBP-TTC-SOD. The active recombinant proteins were purified and, like fragment C alone, bound to rat primary dorsal root ganglion cells. The proteins also retained SOD enzyme activity. The proteins were injected intramuscularly into the hind leg or the tongue of mice. Retrograde transport was assessed by visualising the proteins in the spinal cord or the hypoglossal nucleus of the brain stem respectively using light microscopic immunohistochemistry. SOD activity was also measured in the hypoglossal nucleus by ELISA.

Results demonstrate that the fusion proteins undergo retrograde axonal transfer and trans-synaptic transfer. The efficiency of transport of the fusion proteins is comparable to that of TTC alone. This approach may be useful in assessing the therapeutic potential of SOD as well as studying the transport of tetanus toxin and derivatives to the CNS.

Application of botulinum into pretarsal portion of orbicularis in the treatment of blepharospasm.

Aramideh, M., Speelman, J. D., Brans J. W. M., Koelman, J. H. T. M., Bour, L. J. and Ongerboer de Visser, B. W.

Graduate School of Neurosciences, Dept. of Neurology and Clinical Neurophysiology, Academic Medical Centre, Amsterdam.

Botulinum neurotoxin (BoNT) is the causative agent of the severe neuroparalytic disease botulism. BoNT is produced by toxigenic strains of the species *Clostridium botulinum* as a progenitor toxin complex. The complex consists of BoNT associated with at least one non-toxic component. Sequencing of the region upstream of BoNT from different toxin types has revealed the presence of genes encoding components of the neurotoxin complex. The gene encoding the nontoxic-nonhemagglutinin component (NTNH) is present directly upstream of BoNT in all antigenic types. We report the sequence of a previously unidentified open reading frame (orf) upstream of NTNH found in certain strains of Clostridium botulinum, initially located by East and Collins (1994). The orf encodes a protein (P-47) of ~47 kDa. Attempts to identify the gene in other clostridial strains and analysis of possible function will be discussed.

Reference: East and Collins, 1994, Current Microbial. 29, 69-77.

Botulinum toxin in the treatment of IV nerve chronic palsies.

Buonsanti, J. L. and Rivero Sanchez-Covisa, M. E.

Fundacion Oftalmologica Argentina Jorge Malbran, C/ Parera 15, 8í piso, 1014 - Buenos Aires, Argentina.

In this paper we try to show if botulinum toxin could be used in fourth nerve chronic palsies treatment modalities. We have treated 12 patients with chronic palsies, aged 28 to 88 years of age who had superior oblique palsy with botulinum toxin (BTX) injections into the homolateral inferior oblique. All cases were unilateral. We localised the inferior oblique with electromyographic (EMG) recording. Four cases were injected a second time. The follow-up was from 17 to 29 months. Of the 12 patients, 7 (58%) had excellent results, 2 (17%) good and 3 cases (25%) poor. Transient spread of paralytic effect to the inferior rectus occurred in 4 patients. Botulinum toxin is a useful drug to treat chronic partial superior oblique palsy.

Botulinum toxin in cervical dystonia : effectiveness of low dosage.

Brans, J. M. W., de Boer, I. P., Aramideh, M. and Speelman, J.

Department of Neurology, Academic Medical Centre, Amsterdam, Netherlands.

Botulinum toxin type A (BTA) has been proved to be an effective and safe therapy for cervical dystonia. in contrast to others, we inject a lower dosage of BTA, always under EMG-guidance and we more often inject the lateral (scalenus) and deep cervical (semispinalis) muscles. Aim of this study is to compare our effectiveness with that of previous reports.

Sixty patients with idiopathic cervical dystonia received 240 treatments with BTA (Dysport®). Muscles were selected according to the pattern of movements, hypertrophia and EMG activity. The clinical effect was measured at the moment of reinjection by the Tsui-scale and a 10-points anchored visual analogue scale (self-assessment). A clinically relevant improvement was defined as a decrease of at least 3 points (Tsui) and 2 points (self-assessment), compared to the base-line score.

A dosage of 150 to 300 U was used in 77% of the treatments (mean 204 U). The mean interval was 88 days. Based on the Tsui-scale, 45% of 240 treatments were still effective at the moment of reinjection (median 2 points). Based on the 10-points scale, 73% of the treatments were successful (median 3 points). Eighty percent of 60 patients responded favourably. The side effects were mild. Dysphagia occurred in 9%. A striking difference to previous reports using Dysport® is the lower dosage used in this study. The clinical response, however, is similar to other reports. We conclude that a dosage of 200 to 400 U may also be effective in the treatment of cervical dystonia. An explanation for this may be the use of EMG-guidance and application of BTA into deeply located cervical muscles.

Pharmacologic characterisation of botulinum toxin for basic science and medicine.

First, E. R., MacCallum, R. D., Gupta, A. and Pearce, L. B.

Associated Synapse Biologics, 11 Hurley Street, Cambridge, MA 02141. USA.

The use of Botulinum neurotoxin (BoNT) is increasing in both clinical and basic science. Basic scientists, in particular neuroscientists, are using BoNTs as tools to develop a better understanding of the mechanisms underlying the neurotransmitter release process. Consequently, our ability to accurately and reliably quantify the biologic activity of botulinum toxin has become more important than ever. To develop a better understanding of the methods and units of biologic activity used to characterise the toxin a review of this area was undertaken and is summarised in this poster. This review encompasses current concerns as well as the history of toxin measurement from the 1800's when assays involved feeding raw contaminated food to animals inducing botulism, to present day study of the sub-cellular enzymatic effects of the toxin on synaptic proteins. Analysis of these reports illustrates that there is no single assay method that is most correct for the measurement of activity and that the choice of method is dependent upon the context in which the toxin is being studied or employed. The material presented in the poster will be complemented by a computer program that will allow viewers to access interactive timelines and tables, a searchable database, and other tools to quickly search the history and development of BoNT.

Efficacies of various types and preparations of botulinum neurotoxins - therapeutic consequences.

Güschel, H., Schneck, K., Frevert, J. and Bigalke, H.

Institute of Toxicology, Hannover Medical School, 30623 Hannover, Germany.

Botulinum toxin Type A (BoNt/A), a neurotoxic protease which cleaves SNAP 25, is one of seven serotypes of botulinum toxins and widely used in the treatment of muscular dystonias. Most patients, especially those with focal forms of the disease, benefit by local injections into the rigid muscle, and the resultant recovery of muscle function continues for several weeks to months. Those patients, however, who form specific antibodies during long-term treatment, must abandon the drug probably for the rest of their lives. Since other serotypes of BoNt show similar specificity for the neuromuscular junction and lack cross-immunogenicity, these may be useful alternatives in the therapy of antibody-forming individuals. Each toxin has its own substrate with a single specific cleavage site. Although the substrates are involved in exocytosis in all mammals, there are species-dependent differences in toxicities. This may be due not only to differences in the pharmacodynamics but also in the kinetics in various species. Generally, efficacies and potencies of toxin preparations are tested in mice and expressed as mouse lethal dose (MLD), with one MLD being equivalent to one unit (U). Since respiratory failure terminates toxicity tests in animals, it is preferable to perform the test in the isolated respiratory muscle, avoiding the complex pharmacokinetics in the whole animal. The mouse N. phrenicus-hemidiaphragm preparation has proved to be a useful tool to test toxicities accurately and in a reproducible manner. The two commercial toxin preparations used, which both consist of the neurotoxic compound and hemagglutinins, mimic the dose response curve of the purified neurotoxin A with respect to MLD. BoNt/B, which cleaves synaptobrevin II, is as toxic as BoNt/A. Despite the difference in substrates of types A and B, the combined toxins act additively. Type C_1, whose substrate, syntaxin, forms the fusion complex with SNAP 25 and synaptobrevin II, is far less effective. We suggest that, in cases of specific antibody production, type B could serve as an efficient substitute for A. Moreover, the therapeutic use of a combination of types A and B would reduce the dose of each immunogenic protein while sustaining the paralytic effects. Unwanted antibody production could thus be minimised

Production and characterisation of monoclonal antibodies to a novel trypsin-generated epitope at the C-terminus of the L-H_N of BoNT/A.

Brosnan, M. P., Fooks, S., Hallis, B., Shone, C. C. and Quinn, C. P.

Research Division, CAMR, Porton Down, Salisbury, Wilts. SP4 0JG.

Synthetic peptides corresponding to the putative C-terminus of the trypsin generated L-HN of botulinum neurotoxin type A (L-HN/A) were used as antigens to produce murine monoclonal antibodies. The immunoglobulins produced by the hybridoma cell lines were characterised with respect to their reactivity in ELISA to native BoNT/A, formalin toxoided BoNT/A and the L-HN/A. Antibodies were demonstrated to be reactive against only the L-HN/A establishing that (i) the C-terminal end of the HN constitutes an epitope, (ii) this epitope comprises at least part of the predicted trypsin cleavage site for BoNT/A and (iii) the epitope is not present on the full length toxin

Characterisation of human single chain Fv (scFv) antibody fragments against botulinum neurotoxin type A, B, C and E from immune and non-immune phage display libraries.

Amersdorfer, P., Smith, T., Sheets, M. and Marks, J. D.

Department of Anaesthesia and Pharmaceutical Chemistry, University of California, San Francisco. CA 94110. USA.

Botulinum neurotoxins, which cause the flaccid paralysis associated with the disease botulism, are proteins composed of two polypeptide chains. The light chain possesses the enzymatic activity and the heavy chain is responsible for binding to neuronal membranes. The carboxy-terminal half of the heavy chain (H_C) mediates neurospecific binding and the amino-terminal half of the heavy chain (HN) assists in internalisation of the toxin. To produce human antibodies capable of toxin neutralisation, we created a $7.7x10^5$ single chain Fv (scFv) phage antibody library from a human volunteer immunised with botulinum pentavalent toxoid (ABCDE). Hybridisation of the unselected library using immunoglobuline-Vlambda (Vλ) or Vkappa (Vκ) light chain specific primers, demonstrated a ratio of 66% Vκ light chain genes and 33% Vλ light chain genes, which is similar compared to the natural VL repertoire in humans. Phage expressing scFv which bound Botulinum neurotoxins were selected by panning the phage library on immobilised BoNT A, BoNT B, BoNT C, BoNT E or BoNT A C-fragment (H_C). A total of 23 scFv were isolated which bound BoNT A: 5 of these bound the H_C domain and 4 bound the H_N domain. At least 17 scFv were isolated against BoNT B, 6 scFv were isolated against BoNT C, 3 scFv were isolated against BoNT E and 1 scFv was isolated against BoNT A C-fragment. All scFv were highly specific for the serotype used for selection, with no cross reactivity observed against other serotypes. In a separate set of experiments, a large non-immune human scFv phage antibody library ($7.0x10^9$) was selected on BoNT A, BoNT B, BoNT C, BoNT E and BoNT A C-fragment. Again, binding scFv were obtained against all serotypes, Differences between results obtained from the 2 libraries were observed with respect to V-gene usage and serotype cross reactivity. We are currently working on the expression of soluble scFv in *E. coli* for neutralisation studies in vitro and vivo.

Antibody titres in Botulinum A toxin treated patients and botulinum toxoid-immunised scientists.

Wohlfahrt, K., Dengler, R., Göschel, H. and Bigalke, H.

Department of Neurobiology, Medical School of Hanover, 30623 Hannover, Germany.

Treatment of focal dystonias with botulinum toxin type A is, at times, unsuccessful. This may be due to specific antibodies some patients produce after long-term treatment. Therefore, we have tested the sera of 60 patients injected repeatedly with botulinum toxin as well as the sera of 38 untreated volunteer blood donors and 5 scientists immunised with pentavalent botulinum toxoid 10 years ago. Titres of anti-neurotoxin antibodies were determined by a highly sensitive neutralisation test (mouse N. phrenicus-hemidiaphragm), and anti-hemagglutinin antibodies were measured by ELISA. Not one of the volunteers had specific antibodies. On the other hand, all of the scientists exhibited high titres of anti-neurotoxin antibodies (0.05 IU/ml). In view of the long boost-free period the sustained immunity is remarkable. In the patients group two secondary non-responders showed borderline antibody levels (<0.001 IU/ml), whereas a third non-responder had a high anti-neurotoxin antibody level (>0.05 IU/ml). Moreover, one primary non-responder exhibited a moderate titre of anti-neurotoxin (0.01 IU/ml). None of the successfully treated patients had produced anti-neurotoxin antibodies, although two of them had anti-hemagglutinin antibodies. It appears that failure of therapy with botulinum toxin is due to anti-neurotoxin antibodies. In contrast, anti-hemagglutinin antibodies have no toxin-neutralising activity. Since the antibody titres persist in sera of immunised scientists over a number of years, the prospect for patients with anti-neurotoxin antibody titres is bleak. However, it is conceivable that the other serotypes of botulinum toxins might serve as useful substitutes.

Isolation and characterisation of mouse monoclonal antibodies which bind botulinum neurotoxin type A.

Wong, C. W., Smith, T. and Marks, J. D.

Department of Anaesthesia and Pharmaceutical Chemistry, University of California, San Francisco. CA 94110. USA.

Clostridium botulinum neurotoxins (BoNTs) are neurotoxic proteins that block the release of the neurotransmitter acetylcholine resulting in the neuroparalytic disease, botulism. In order to produce neutralising antibodies (Ab), we created a 2×10^6 member single chain Fv (sFv) phage antibody library from mice immunised twice with the BoNT type-A C-fragment (cellular receptor binding domain) and then once with the BoNT type-A. Restriction endonuclease fingerprinting of phage DNA from the library indicated that 90% of the recombinant phage contained a sFv insert and that the library appeared to be diverse. Phage antibodies were selected either on intact BoNT type-A or on the BoNT type-A C-fragment. More that 30 unique sFv were isolated by selecting on the complete toxin. Only four of these recognised the C-fragment. At least 15 unique sFv were identified by selecting on the C-fragment, 50% of which also bound the complete toxin. Based upon the DNA sequences obtained from the selected sFvs, we believe it is likely that the sFvs bind to different epitopes on the type A BoNT. We conclude that immune phage Ab libraries are a powerful technique for producing large numbers of different Ab, thus increasing the chances of isolating neutralising Ab. Presently we are expressing soluble sFv in *E. coli* for kinetic studies and for neutralisation studies in vivo.

Development of a genetically engineered vaccine for botulinum toxin.

Smith, L. A.

USAMRIID, Toxinology Division, Fort Detrick, Frederick, MD. 21702-5011. U.S.A.

Botulinum neurotoxins (BoNT) are the most potent biological agents discovered to date (mouse lethal dose < 0.1 ng kg^{-1}). Seven immunologically distinguishable forms of these neuroparalytic toxins, designated as types A, B, C1, D, E, F and G, are produced by different strains of the anaerobic, spore-forming eubacterium, *Clostridium botulinum*. Immunity elicited against one of the serotypes will not protect from any other serotype. Because of the numerous limitations associated with the current pentavalent botulinum toxoid vaccine, we are developing a new-generation recombinant vaccine. Synthetic genes encoding non-toxic 50 kDa carboxy-terminal regions of the toxin (referred to as fragment C or Hc) were constructed for serotypes A, B, E and G. Genes specifying BoNT(Hc)A, B and E were expressed in *E. coli* and in the yeast, *Pichia pastoris*. Vaccination of mice with monovalent recombinant A-and B-Hc protected the animals from a challenge of up to 10^6 MLD50 of botulinum toxin A and B, respectively. Protection studies using E-Hc and a combinational trivalent vaccine are in progress.

Delivery of BoNT/A to neuroblastoma cells in vitro using recombinant antibodies.

Quinn, C. P. and Shone, C.C.

Research Division, CAMR, Porton Down, Salisbury, Wilts. SP4 0JG.

Gene sequences coding for the single chain fraction variable (ScFv) of monoclonal antibodies reactive against the HC domain of botulinum neurotoxin type A (BoNT/A) were cloned using a phage display system and expressed in E. coli as a soluble peptide fused to a synthetic IgG binding domain (ZZ). The antibodies (ZZ-ScFv) were demonstrated by ELISA to possess both IgG and BoNT/A reactivity in both the solid and aqueous phase. ZZ-ScFv constructs were also demonstrated in vitro to deliver 125I-BoNT/A to the surface of SH-SY5Y neuroblastoma cells at $+4°C$ in the presence of OX-26 monoclonal antibody to the transferrin receptor.

A capillary electrophoresis-based assay of Botulinum type A toxin proteolytic activity.

Corran, P. H. and Sesardic, D.

Division of Bacteriology, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Herts. EN6 3QG.

The light chains of tetanus and all botulinum neurotoxins possess a highly specific Zn^{++} dependent proteolytic activity directed at one or other of the components of the synaptic vesicle docking system (responsible for exocytosis of neurotransmitters). In the case of Botulinum neurotoxin Type A (BoNT/A) the target is SNAP25 (Synaptic Membrane Associated Protein), which is cleaved at the bond Q197-R198, to release a 9 residue fragment from the C-terminal. We have used a synthetic 70 residue peptide consisting of an extended C-terminal fragment of SNAP25 (SNAP25137-206) which is a substrate for BoNT/A (courtesy of Dr C Shone, CAMR). The proteolytic cleavage of SNAP25137-206 could be followed by Capillary Zone Electrophoresis. Disappearance of SNAP25137-206 was accompanied by the appearance of components with mobilities corresponding to those calculated for the expected products, SNAP25137-197 and SNAP25198-206. The reaction was inhibited by EDTA. Since the entire 137-206 sequence is devoid of aromatic amino acids, the reaction was monitored using the UV adsorption of the peptide bond at low wavelength (200 nm). Under these circumstances the sensitivity is greatest using the formation of SNAP25137-197 or disappearance of SNAP25137-206 (the 9 residue C-terminal fragment has few peptide bond chromophores). Several assays may be carried out simultaneously by automating the process of sequential repetitive sampling of the reaction mixtures. The sensitivity is adequate for detecting and measuring BoNT/A activity in clinical preparations. Using this assay, three formulated preparations were ranked in order of potency, although the relative rates of reaction did not correspond exactly to the relative biological potencies.

A rapid and sensitive fluorescent enzymatic assay for tetanus toxin light chain metallopeptidase activity.

Martin, L., Cornille, F., Soleilhac, J-M., Fournie-Zaluski, M-C. and Roques, B. P.

Département de Pharmacochimie, Moléculaire et Structurale, U 266 INSERM, UFR des Sciences Pharmaceutiques et Biologiqes, 4, Avenue de l'Observatoire - 75270 PARIS Cedex 06, France.

Tetanus toxin (TeNT) is constituted by a heavy chain (H chain, 100 kDa) and a light chain (L chain, 50 kDa), held together by a disulphide bridge. The light chain, endowed with a zinc metalloendopeptidase activity, cleaves specifically the vesicle-associated membrane protein (VAMP, also called synaptobrevin, at a single peptide bond (G1n76-Phe77), resulting in the blockade of neuroexocytosis pathway. We report here an enzymatic assay allowing the screening of selective and potent inhibitors of this neurotoxin, such compounds being unknown at this time. For this purpose the purification of the light chain of the toxin (TeNT-L chain) was achieved by a rapid and convenient procedure using ion-exchange chromatography, leading to a dissociation of subunits without loss of activity. The 50-mer peptide corresponding to the synaptobrevin sequence 39-88 (S 39-88) was synthesised by solid phase method and was found to be the minimum substrate of TeTx. Taking advantage of the difference in lipophilicity of the substrate and the short metabolite S 77-88, we introduced in the latter the strongly efficient fluorophore pyrenylalanine (Pya) by substitution of Tyr88. This non natural amino-acid was synthesised in good yields by an enantioselective method. The single fluorescent metabolite (Pya88) S 77-88 formed by action of TeNT on the substrate (Pya88) S 39-88 was easily separated from the substrate in one step by use of SEP-PAK Cartridges and quantified by fluorescence emission at 377 nm allowing the detection of nanomolar concentration of this metabolite. Moreover, this fluorescent substrate was determined to be 4 times more potent towards the TeNT-L chain than S 39-88 (Kcat/Km = 9635 M-1 min-1 and 2455 M-1. min-1 respectively). This novel enzymatic assay, which could be easily extended to other neurotoxins, represents a large improvement in terms of sensitivity and timesaving as compared to methods currently used (SDS-PAGE, HPLC).

Standardisation of botulinum toxin type A used clinically: laboratory investigations and international collaborative study.

McLellan, K., Gaines Das, R. and Sesardic, D.

National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Herts. EN6 3QG.

Clinical formulations of Botulinum type A toxin have been licensed for the treatment of muscle disorders for several years. Problems have been identified in the area of potency testing for control purposes, in particular when comparing potency estimates of products supplied by different manufacturers. The specific activity of therapeutic preparations is currently determined using the mouse lethality assay and is expressed as mouse LD50 units. Clinical use has highlighted discrepancies in labelled mouse LD50 units between different formulations. This is caused by implementation of different assay systems. In agreement with others we have shown that buffers, method of sample preparation and end-point can significantly affect absolute potency estimates. In our laboratory a comparison of saline and gelatine phosphate buffer (GPB), used for reconstitution and dilution, demonstrated a significant shift in toxin activity, with two different preparations each being on average twice as potent in GPB than in saline when the same nominal amount of toxin was administered. A significant shift in activity (30%) is also observed for absolute potencies obtained at 24 and 48 hours post injection. These observations highlight the need for a standardised unit of measurement, and hence the introduction of a reference material. NIBSC is currently co-ordinating an international collaborative study on standardisation of Botulinum type A toxin, which aims to assess the suitability of three different candidate standards for use as the First International Standard for Type A Toxin. An additional objective of the study is to assess and compare the currently used assay methods for measurement of biological activity of type A toxin used clinically.

The development of novel assays for the detection and quantitation of botulinum neurotoxins.

Newton, K., Wictome, M. and Shone, C.

Kirsti Newton, Research Division, CAMR, Porton Down, Salisbury. SP4 0JG.

The seven related toxins produced by *Clostridium botulinum* act on the neuromuscular junction where they block release of the neurotransmitter acetylcholine. Their action results in the life threatening condition "botulism", characterised by widespread flaccid muscular paralysis.

To insure food safety and diagnose outbreaks of botulism a rapid and sensitive assay is required to detect the neurotoxin in food samples. The current assay – the mouse lethality test – has a number of disadvantages. The assay is expensive, does not determine the toxin type and takes up to four days to perform. A large number of animals are also required if the neurotoxin is to be accurately quantified; whilst the use of animals in such assays is becoming increasingly socially unacceptable.

Recently the molecular mode of action of the toxins has been elucidated, demonstrating that the toxins possess highly specific protease activities against proteins involved in the release of neurotransmitter from the synapse. Their enzymic action has been used to develop microtitre based assays which are rapid, quantitative and determine the toxin type whilst retaining the sensitivity of the current mouse lethality test.

Different sensitivities of neuro-endocrine cells in culture to externally applied botulinum neurotoxins.

Duggan, M. J., Doward, S., Boyd, R. S., Jen, A. Y., Burns, L., Purkiss, J. R., Welch, M. J. and Foster, K. A.

Research Division, CAMR, Porton Down, Salisbury, Wilts. SP4 0JG.

The identity of most of the cell-surface receptors for the botulinum neurotoxins (BoNT) remains unknown. One approach to identifying the receptors and testing candidate receptors is to compare the relative sensitivities of different cell types to BoNT.

We have, therefore, established a number of different cell culture systems of neuro-endocrine origin. These include established cell lines such as PC12 (phaeochromacytoma), SHSY-5Y (neuroblastoma), RINm5f (insulinoma); and primary cultures such as adrenal chromaffin cells and dorsal root ganglion neurons.

These cells express the BoNT substrates (SNAP-25, synaptobrevin, and syntaxin). Treatment of these cells with various BoNT serotypes results in proteolysis of the substrates. In some cell types we have shown that secretion of the appropriate hormone or neurotransmitter is inhibited.

These cultured cells show a differential sensitivity to BoNT. As expected the primary neuronal cultures were the most sensitive, however, the established cell lines also differed in their profile of sensitivity.

Importance of two adjacent C-terminal sequences of SNAP-25 for exocytosis from intact and permeabilised chromaffin cells revealed by inhibition with botulinum toxin A and E.

[1]Lawrence, G. W., [1]Foran, P., [2]DasGupta, B. R. and [1]Dolly, J. O.

[1]Dept. of Biochemistry, Imperial College, London SW7 2AY, U.K. and [2]Dept. of Food Microbiology and Toxicology, Food Research Institute, University of Wisconsin.

Types A and E botulinum toxin (BoNT) inhibit neurotransmitter release by similar mechanisms. Both are Zn^{2+} -requiring endoproteases which enter nerve terminals and cleave a synaptosomal-associated protein of Mr = 25 kDa (SNAP-25), but at different peptide bonds; BoNT/E removes 26 residues from the C-terminal whereas BoNT/A cleaves off only 9 amino acids. As SNAP-25 is also expressed in chromaffin cells, its BoNT-susceptibility was examined in relation to blockade of exocytosis from large dense-core granules. Here, we report a dose-dependent cleavage of SNAP-25 by BoNT/E; this correlates with its inhibition of Ca^{2+} -triggered catecholamine release from chromaffin cells. Near-complete blockade of secretion and proteolysis of SNAP-25 were achieved with 100 nM BoNT/E, indicating the importance of this protein for exocytosis from neuroendocrine cells. Notably, truncation of all of the SNAP-25 in intact cells by exposure to BoNT/A resulted in only partial blockade of the exocytotic response to Ca^{2+} after digitonin-permeabilisation, despite evoked exocytosis from intact cells having been abolished. Importantly, the BoNT/A-resistant release was eradicated by adding BoNT/E to the permeabilised cells; it further truncated the SNAP-25 fragment that had been produced by BoNT/A. When cells were permeabilised and then exposed to either toxin, both types blocked MgATP-dependent secretion but only BoNT/E attenuated the energy-independent phase. These distinct inhibitory effects of the two toxins demonstrate that residues 198-206 of SNAP-25 are absolutely essential for exocytosis from intact cells whereas even in their absence a significant proportion of the exocytotic response can be elicited from permeabilised cells, but this is totally reliant on amino acids 180-197. This demonstrated participation of BoNT/A-truncated SNAP-25 in catecholamine release, observed under artificial conditions, accords with its reported ability to complex with other exocytosis proteins and highlights the importance of such interactions. However, it is unclear why the decreased stability of such complexes with BoNT/A-cleaved SNAP-25, observed by others in vitro, permits some exocytosis in permeabilised but not intact cells.

Botulinum toxin C1 cleaves both syntaxin and SNAP-25 causing inhibition of catecholamine release from intact and permeabilised chromaffin cells.

[1]Foran, P., [1]Lawrence, G., [2]Shone, C. C., [2]Foster, K. and [1]Dolly, J. O.

[1]Dept. of Biochemistry, Imperial College, London SW7 2AY, U.K. and [2]CAMR Porton Down, Salisbury, Wilts. SP4 0JG

The seven types (A-G) of botulinum neurotoxin (BoNT) are Zn^{2+} -dependent endoproteases that potently block neurosecretion. Syntaxin was thought to be the sole neuronal substrate for BoNT/C1; synaptosomal-associated protein of Mr =25 000 (SNAP-25) is selectively proteolyzed by types A and E at Q197 - R198 and R180 -I181, respectively. As SNAP-25 has been shown to be crucial for exocytosis in neuroendocrine cells, evidence was sought herein for the involvement of syntaxin IA/B in the exocytosis of dense core granules from adreno-chromaffin cells. Intact cells were incubated with BoNT/C1 at various concentrations; Western blotting of a membrane fraction from these intoxicated cells demonstrated a good correlation between cleavage of syntaxin 1A/B and the observed reduction in Ca^{2+} - or Ba^{2+} -dependent catecholamine secretion, with no change in the contents of other plasma membrane (GAP-43) or granule (synaptotagmin, synaptobrevin, cellubrevin) antigens. However, blotting with antibodies against a C-terminal peptide of SNAP-25 revealed the additional disappearance of immunoreactivity, with the same toxin concentration dependency as syntaxin breakdown. The product of SNAP-25 cleavage by C1 was similar in size to that produced by A; however, contamination of BoNT/C1 by serotypes A or E was eliminated. Notably, C1 treatment of chromaffin cells abolished Ca^{2+} -evoked catecholamine secretion following digitonin-permeabilisation compared with a partial inhibition exerted by BoNT/A, establishing the importance of syntaxin 1A/B for exocytosis. Unexpectedly, C1 failed to proteolyse a soluble form of recombinant SNAP-25 that served as a good substrate for BoNT/A. These interesting observations* suggest that BoNT/C1 can only cleave SNAP-25 when it is an unique conformation and/or associated with other factors present in the cells.

*Foran et al., (1996) Biochemistry 35, 2630-2636.

Description of an open reading frame found in the botulinum neurotoxin operon structure in some strains of Clostridium botulinum.

Bhandari, M. East, A. K., and Collins, M. D.

Microbiology Department, Institute of Food Research, Earley Gate, Whiteknights Road, Reading, RG6 6BZ. UK.

Botulinum neurotoxin (BoNT) is the causative agent of the severe neuroparalytic disease botulism. BoNT is produced by toxigenic strains of the species *Clostridium botulinum* as a progenitor toxin complex. The complex consists of BoNT associated with at least one non-toxic component. Sequencing of the region upstream of BoNT from different toxin types has revealed the presence of genes encoding components of the neurotoxin complex. The gene encoding the nontoxic-nonhemagglutinin component (NTNH) is present directly upstream of BoNT in all antigenic types. We report the sequence of a previously unidentified open reading frame (orf) upstream of NTNH found in certain strains of Clostridium botulinum, initially located by East and Collins (1994). the orf encodes a protein (P-47) of ~47 kDa. Attempts to identify the gene in other clostridial strains and analysis of possible function will be discussed.

Reference: East and Collins, 1994, Current Microbial. 29, 69-77.